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STEMPHYLIUM LEAF SPOT OF RED CLOVER AND ALFALFA[1]

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INTRODUCTION

One of the foliage diseases of red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) is caused by a fungus formerly known as a *Macrosporium*, but more recently as a species of *Stemphylium*. The causal fungus, which is characterized by echinulate conidia and has an ascigerous stage belonging in the genus *Pleospora*, has been known previously as a parasite of red clover and alfalfa, but unfortunately has sometimes been confused with *Macrosporium sarcinaeforme* Cav., a fungus that has smooth-walled conidia, has no known ascigerous stage, and is known to occur only on red clover in nature. The investigation reported in this paper was designed to trace the life history of the echinulate-spored fungus and to clarify any confusion that may exist in the literature regarding its identity and relationship to *M. sarcinaeforme* and other similar fungi on red clover and alfalfa.

Krakover (*9*)[2] and Horsfall (*6*) have shown that the fungus on red clover has smooth-walled conidia and corresponds very well with Cavara's original description of that species. Wiltshire (*24*) has transferred this fungus to the genus *Stemphylium*, and according to him, it should be known as *S. sarcinaeforme* (Cav.) Wiltshire. Gentner (*5*) reported the echinulate-spored fungus to be the cause of a disease of both red clover and alfalfa in Germany, but he misidentified it as *Macrosporium sarcinaeforme*. He also reported perithecia of *Pleospora herbarum* (Pers. ex Fr.) Rabh. in pure cultures of this echinulate-spored fungus. The association of this ascomycete with a fungus having sarcinaeform, warty conidia was shown by the Tulasne brothers (*21*) in 1863 and has since been confirmed by several other investigators. This work has been reviewed by Wiltshire (*24*). In New York, Horsfall (*6*) obtained single-ascospore cultures of what he thought was *P. herbarum* from overwintered red clover stems two seasons in succession. He reported that these cultures were identical in every respect with those of the *Macrosporium* from lesions on alfalfa leaves, including the production of spiny conidia. He suggested, therefore, that the *Macrosporium* from alfalfa and its possible sexual stage, *P. herbarum*, may occur on both red clover and alfalfa, but that *M. sarcinaeforme* from red clover is limited to that suscept in nature. In the present paper the spiny-spored fungus is reported to be pathogenic on both alfalfa and red clover and is identified as *Stemphylium botryosum* Wallr.

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[2] Italic numbers in parentheses refer to Literature Cited, p. 845.

The ascigerous stage has been found on dead red clover stems and has developed on sweetclover stems and on potato-dextrose agar. It is identified as *Pleospora herbarum*. *P. denotata* (Cke. and Ell.) Sacc., originally described from dead stems of red clover and differing from *P. herbarum* only in the larger size of the ascospores, is considered by the writer to be a synonym of *P. herbarum*.

THE DISEASE

On red clover the disease occurs mainly on leaf tissue but it may also occur on stems and petioles. Leaf lesions are irregular in shape and dark brown to black in color (fig. 1, *A* and *B*). Around each

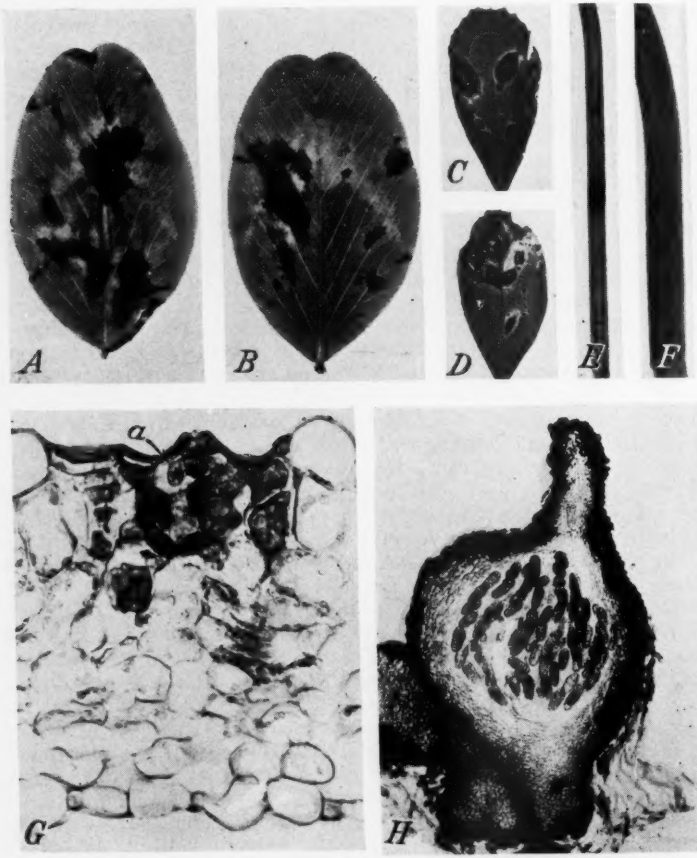

FIGURE 1.—*A* and *B*, Leaves of red clover artificially inoculated with crushed perithecia of *Pleospora herbarum*, about natural size; *C* and *D*, natural infection of alfalfa leaves by *P. herbarum*, about natural size; *E* and *F*, petiole and stem of red clover artificially inoculated with crushed perithecia of *P. herbarum*, × about 1½; *G*, early stage of infection of red clover leaf by *P. herbarum* showing enlarged mycelium (*a*) in substomatal cavity, ×about 430; *H*, cross section of a perithecium of *P. herbarum* grown on a sweetclover stem, ×about 100.

lesion a straw-colored halo often develops and maintains itself in advance of the enlarging lesion. Under favorable conditions the lesions increase in size quite rapidly and coalesce until much of the leaf is involved. Under field conditions the disease is difficult to diagnose on stems and petioles, as it is often associated with the black stem disease caused by *Phoma trifolii* Johnson and Valleau (*8*). However, small black linear lesions are produced on stems and petioles following inoculation with pure cultures of the causal organism (fig. 1, *E* and *F*). On stems these lesions have never developed extensively, and it appears unlikely that such infections are capable of doing appreciable damage to the plant. On petioles, however, the lesions sometimes develop sufficiently to cause breaking at the locus of infection.

PATHOLOGICAL HISTOLOGY

A modification of the cleared-leaf method described by Peace (*13*) was used for observing spore germination and early stages of infection on leaf tissue. Bits of leaf tissue were cleared in alcohol-acetic acid 50–50, stained with cotton blue in lactophenol, then mounted in clear lactophenol. For more detailed observation on early stages of infection and host-parasite relations, stained microtome sections were made of infected leaf tissue. The tissue was fixed in Karpechenko's modification of Navaschin's fixing fluid, consisting of equal parts of solution A (195 cc. water, 30 cc. glacial acetic acid, and 3 gm. chromic acid) and solution B (195 cc. water, 30 cc. formalin (37 percent formaldehyde)) mixed just before using. The material was dehydrated and infiltrated with paraffin, according to the schedule given by Rawlins (*16*), by the use of cedar oil following a graded series of alcohols up to 95 percent. Sections were cut 10μ and 15μ in thickness. Staining with safranin and fast green gave satisfactory differentiation between host and parasite.

Since spore germination and penetration of the mycelium into the leaf are similar for both ascospores and conidia, the following description of these processes applies to both. Illustrations were drawn only for ascospores. Spores germinate from several of the cells that comprise the spore (fig 2, *A*). Penetration into the leaf is usually through stomata but may be directly between cells. Usually a strand of mycelium grows over the leaf surface until it reaches a stoma, becomes somewhat swollen over the opening, and an infection hypha enters the substomatal cavity (fig. 2, *B*). Cases have been observed, however, where hyphae have grown directly across stomata without entering. In the substomatal cavity the fungus usually forms two or three short, thick strands of mycelium, about two or three times as thick as aerial mycelium or mycelium subsequently formed within the host tissue (fig. 2, *B*). This bulbous mycelium apparently makes very little growth until several host cells immediately around the stoma have been killed (fig. 1, *G*); it then continues to grow intercellularly through the leaf tissue as fine strands of mycelium (fig. 2, *D*).

In the few cases of direct penetration that were observed, a germ tube, about twice the thickness of other germ tubes, had grown only a short distance from the spore and had then penetrated the leaf between epidermal cells (fig. 2, *C*). After gaining entrance into the leaf the fungus grows intercellularly through the leaf, killing the host cells as it progresses. During the early stages of infection, very few

strands of mycelium were observed inside the leaf in either microtome-sectioned material or in cleared leaves stained with cotton blue. Conidiophores and conidia were produced on leaf lesions in about 5 to 8 days after inoculation (fig. 2, *E*).

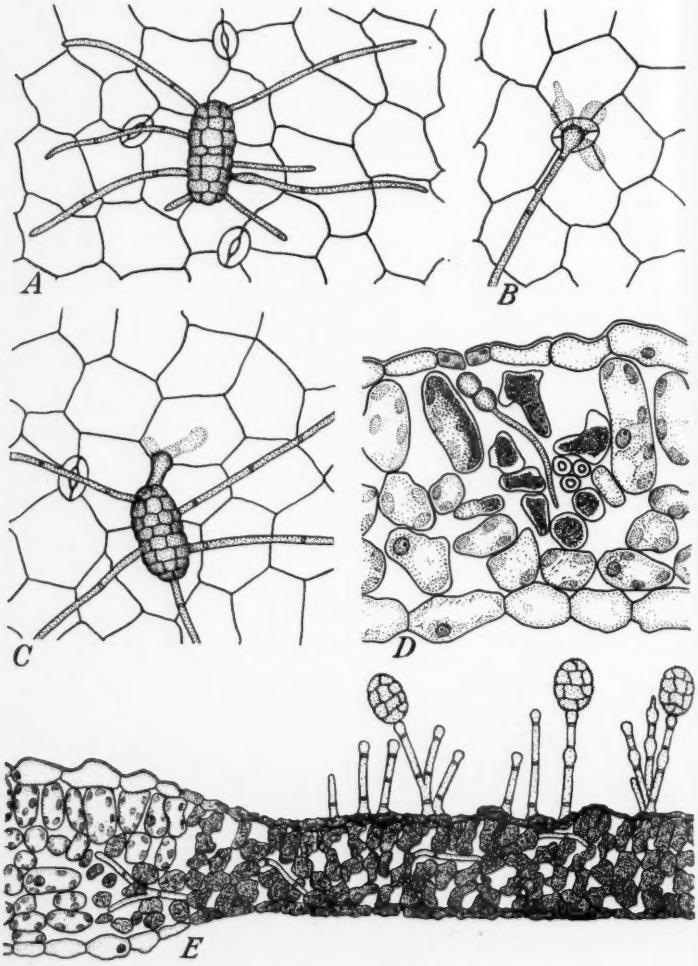

FIGURE 2.—Camera-lucida sketches of *Pleospora herbarum* on red clover leaf tissue: *A*, Germinating ascospore, × about 420; *B*, early stage of stomatal penetration showing enlarged hyphae in substomatal cavity, × about 420; *C*, early stage of direct penetration, × about 420; *D*, early stage of infection, showing enlarged mycelium in substomatal cavity and normal-sized mycelium in region of mesophyll and spongy parenchyma, × about 420; *E*, leaf lesion showing conidiophores and conidia, × about 275.

PATHOGENICITY OF THE FUNGUS

The organism is readily isolated from field material by placing small pieces of surface-sterilized diseased leaf tissue on acidified potato-dextrose agar. From characteristic leaf lesions pure cultures of the organism are obtained in nearly every case. Although isolates of the organism differ slightly in color they are usually white during the first few days of growth, after which the mycelium near the center of the colony develops dark pigments that appear green from the top and dark brown from the under side of the culture. Perithecial primordia begin to appear in the cultures in about 5 to 8 days. It is quite common to find white tufts of mycelium in some cultures, and in a few instances sterile white sectors have occurred similar to those observed by Ellis (*4*) in cultures of *Pleospora herbarum* from *Euphorbia*. While zonation was observed in culture, no attempt was made to determine its cause. Ellis (*4*) attributed the zonation in cultures of *P. herbarum* from *Euphorbia* to temperature variations.

The pathogenicity of the organism was proved by inoculating plants grown in the greenhouse and reisolating the fungus from the lesions produced. Infection was obtained both by spraying the plants with a suspension of conidia obtained from diseased leaves and by smearing a suspension of ascospores and perithecial fragments on the plant with a camel's-hair brush. After all inoculations the plants were kept in a glass-enclosed chamber at a high atmospheric humidity for 2 to 5 days before being removed to a greenhouse bench. Lesions on leaf tissue developed in about 3 to 4 days, whereas stem and petiole lesions were not in evidence until about 10 to 14 days after inoculation.

It was beyond the scope of this investigation to determine the complete host range of this fungus. Inoculations were made, however, on a number of plants during the winter of 1937 with a culture of the organism isolated from diseased red clover leaves collected at Marshfield, Wis. No infection was obtained on the following plants: *Melilotus alba* Desr., *M. officinalis* Lam., *Trifolium incarnatum* L., *T. involucratum* L., *T. agrarium* L., and *T. procumbens* L. Slight infection was produced on *Medicago arabica* Huds., and *T. resupinatum* L. *Medicago sativa* proved to be moderately susceptible. In 1938, inoculations were made on *Melilotus alba* and *Medicago sativa* with a culture of the fungus obtained from diseased leaves of *T. pratense* near Madison, Wis. The organism produced small lesions on *Melilotus alba* and on *Medicago sativa*. Other plants were not available for inoculation at that time. From these limited trials it appeared that different isolates of the fungus differ slightly in pathogenicity, but the organism isolated from *T. pratense* is primarily a pathogen of that plant.

The organism infects the seeds of several leguminous plants. Gentner (*5*) reported it on seeds of red clover, white clover, alsike clover, crimson clover, and lucerne. From F. R. Jones [3] the writer obtained a culture of this fungus isolated by him from living seeds of *Melilotus alba*. Tested by inoculation, it proved to be pathogenic on *M. alba* and *Trifolium pratense*. Thus *M. alba* can now be added to the list of plants that may carry this pathogen in or on the seeds.

[3] Senior pathologist, Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

MORPHOLOGY OF THE FUNGUS

ASCIGEROUS STAGE

The perfect stage of the fungus has been found on dead stems of red clover and has developed in culture on potato-dextrose agar and on sweetclover stems. It is here identified as *Pleospora herbarum*. Perithecia are typically globoid and sometimes possess a slender neck. The latter character is very pronounced on perithecia produced on sweetclover stems but is usually lacking on perithecia produced on potato-dextrose agar. Each fruiting body is usually distinct, has a black membranous wall, and produces many asci (fig. 1, *H*). When the fungus is grown on either potato-dextrose agar or sweetclover stems, perithecia are formed in about 10 to 14 days but asci and ascospores are not produced until about 4 or 5 months later. A temperature of 10° to 12° C. is most favorable for ascospore production. Ellis (*4*) reported that cultures of *P. herbarum* from *Euphorbia* develop perithecia with fully developed asci in about a month, and added (*4, p. 111*): "It is probable that any check to the vegetative growth of the mycelium will be followed by the production of increased numbers of reproductive structures, though all these may not necessarily mature."

Asci are elongate, cylindrical, and typically eight-spored. They measure 183μ to 267μ by 27μ to 37μ, with a mean length of $39\pm0.2\mu$ and a mean diameter of $34\pm0.3\mu$.

Ascospores are muriform and yellowish to brown in color. They are rounded at both ends, have seven cross septa and three to five longitudinal septa. They measure 32μ to 48μ by 14μ to 21μ, with a mean length of $39\pm0.4\mu$ and a mean diameter of $17\pm0.2\mu$. The standard error was calculated from the formula

$$S_x=\sqrt{\frac{\Sigma(x^2)-\frac{(\Sigma x)^2}{n}}{n(n-1)}}$$

Each calculation was based on 100 random measurements.

The method of ascospore discharge is essentially like that described by other investigators (*1, 15, 18*) and will be only briefly reviewed here. Asci are readily liberated from the perithecium by placing the latter on a glass slide in a drop of water, covering it with a cover slip, and applying light pressure. The perithecium ruptures usually at one side of the bulbous portion, and the asci pass out into the surrounding water. The ascus wall consists of two layers, an outer one that is thin and quite firm and an inner one that is thicker and elastic. The two walls are not visibly distinguishable, however, until the outer wall is ruptured during the process of spore discharge.

When an ascus is liberated from the perithecium, it imbibes water and begins to swell (fig. 3, *A*). The outer wall, being inelastic, bursts, usually at the apical end, and contracts slightly, forming several wrinkles or folds around the inner ascus wall (fig. 3, *B*). Simultaneously the inner membrane stretches rapidly and the ascus becomes nearly twice its original length with little or no increase in diameter (fig. 3, *B*).

Asci may elongate immediately on coming in contact with water or several minutes thereafter, but the actual process of elongation requires only 10 to 20 seconds. Immediately after the outer ascus wall bursts and during the elongation of the inner wall, the spores and ascus contents move to the upper portion of the ascus. Usually all the spores except one move above the ring formed by the outer wall. Spores that remain in the lower portion of the ascus are not expelled at the time of spore discharge.

A stretched ascus may discharge its spores immediately or several minutes after it has elongated, or it may elongate and not discharge its

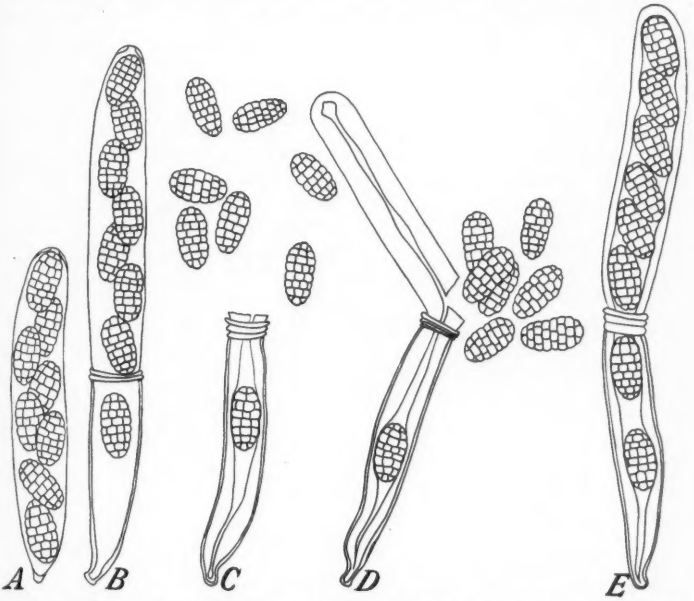

FIGURE 3.—Camera-lucida sketches of ascospore discharge in *Pleospora herbarum*: *A*, Ascus as it appears when first forced from perithecium; *B*, same ascus as *A* after outer wall has burst and inner wall has elongated; *C* and *D*, asci which have discharged their spores; *E*, ascus from which spores were not discharged. Note how the inner wall has become swollen in *C*, *D*, and *E*. × about 240.

spores (fig. 3, *E*). The stretched ascus usually bursts just above the ring formed by the outer ascus wall and the spores are expelled into the surrounding medium. In some cases an ascus bursts on one side and the spores pass out through the rift thus formed (fig. 3, *D*), while in other cases the entire upper portion of the stretched ascus is torn off and the spores are scattered in any direction (fig. 3, *C*). Spores are discharged so quickly that their exit from the ascus cannot be followed with the eye; from observations it appears that they are discharged with considerable force, but because of the resistance offered by the water they remain near the point of release. The inner wall of the ascus thickens considerably soon after stretching and asci that discharge their spores do so before the thickening occurs. Spores that

are not discharged germinate while inside the ascus and the germ tubes grow out through the thickened wall.

CONIDIAL STAGE

The conidial stage of the fungus is considered to be *Stemphylium botryosum* Wallr. (*22*), the species upon which Wallroth founded this genus in 1833. Conidia are rarely produced on potato-dextrose agar, but under conditions of high humidity numerous conidiophores and conidia are produced on leaf lesions. The conidiophores are brown, upright, stout-appearing structures that are either single or grouped in fascicles rarely composed of more than four hyphae. They are slightly bulbous at their bases and also at the distal end where the conidia are borne. They measure 3.5μ to 5.5μ in width but differ considerably in length, depending on environmental conditions and the number of conidia they produce. After a conidium has been produced, the end of the conidiophore grows out, producing new cells and a new conidium. As a result of the continued apical growth, a conidiophore may grow to considerable length and have a nodulose appearance, the swollen cells of the conidiophore indicating the points at which conidia were borne (fig. 2, *E*). Conidia that are not dislodged from the ends of the conidiophore are pushed to one side by the new growth and appear to be borne laterally on the conidiophore.

The conidia are olive brown muriform, and echinulate. They are oblong in shape and often constricted at the center by a median cross septum. They measure 24μ to 40μ by 14μ to 23μ, with a mean length of $31 \pm 0.4\mu$ and a mean width of $19 \pm 0.2\mu$.

There can be no doubt that the perithecial and conidial stages described here belong to the same fungus. On leaf lesions resulting from inoculations with ascospores the fungus produces the echinulate conidia, and cultures from these conidia produce the perithecial stage when grown on sweetclover stems or on potato-dextrose agar. On potato-dextrose agar, cultures started from conidia and ascospores are similar in appearance. On sweetclover stems a few conidia are produced in addition to the perithecia.

The fungus is homothallic. Perithecia and ascospores have been produced on sweetclover stems from either single-ascospore or single-conidium cultures.

TAXONOMY

The fact that fungi similar to the one described above on red clover have been described on several different host plants raises the question whether the red clover organism is identical with one or more of those previously described. Many of the earlier descriptions were of only the conidial or ascigerous stage of the fungus, since no connection was established between the two spore stages. Where possible, cultures of the organisms from the various hosts were obtained and when this material was lacking, herbarium specimens and written descriptions were used. The following described species have been considered: *Pleospora denotata* (Cke. and Ell.) Sacc.; *P. lycopersici* É. and É. March.; *Macrosporium medicaginis* Cugini; *M. sarcinaeforme* Cav. as discussed by Malkoff (*10*), Gentner (*5*), and Hülsenberg (*7*); *M. meliloti* Pk.; and *Thyrospora sarcinaeforme* (Cav.) Tehon and Daniels. A summary of the information obtained regarding most of these fungi is given in table 1.

TABLE 1.—*Summary of information on fungi considered in relation to Stemphylium botryosum*

Fungus	Described or discussed by—	Host	Spore measurements	Character of conidium wall	Ascigerous stage
			μ		
Macrosporium melilati Pk.[1]	Peck (*14*)	*Melilotus alba* Desr.	25–50 long	Smooth	
Macrosporium sarcinaeforme Cav.[1]	Cavara (*2*)	*Trifolium pratense* L.	24–28×12–18	do	
Macrosporium sarcinaeforme Cav.[2]	Malkoff (*10*)	*Trifolium pratense.*	25.2–33.6×16.8–22.4	Echinulate	
Macrosporium medicaginis Cugini.[1]	Traverso (*20*)	*Medicago sativa* L.	25–35×16–18	Smooth	
Macrosporium sarcinaeforme Cav.[2]	Gentner (*5*)	*Medicago sativa* / *Trifolium pratense.*	17–40×8.7–29	Echinulate	*Pleospora herbarum* (Pers. ex Fr.) Rabh.
Thyrospora sarcinaeforme (Cav.) Tehon and Daniels.[2]	Tehon and Daniels (*19*).	*Medicago sativa.*		do	
Macrosporium sarcinaeforme Cav.[2]	Hülsenberg (*7*).	*Medicago sativa.*		do	
Stemphylium botryosum Wallr.	Smith	*Trifolium pratense.*	24–40×14–23	do	*Pleospora herbarum.*
		Medicago sativa.	24–38×14–22	do	*Pleospora herbarum* (*P. denotata* (Cke. and Ell.) Sacc.)

[1] Considered distinct from *Stemphylium botryosum.*
[2] Considered synonymous with *Stemphylium botryosum.*

As will be shown later, most of these organisms are morphologically identical with *Pleospora herbarum* or its conidial stage, *Stemphylium botryosum*, and it appears that, if we are to accept morphological differences rather than small differences in pathogenicity as a basis for making specific differences, then *P. herbarum* and its conidial stage, *S. botryosum*, consist of a group of fungi morphologically alike but possessing some degree of physiological specialization. Wiltshire (*24*) has recently shown that a number of fungi previously described and named in their conidial stage as distinct species are in reality synonymous with *S. botryosum*. He also states that the association of this conidial type with *P. herbarum* as its ascigerous stage was known as early as 1863 by the Tulasne brothers (*21*).

The ascospores of *Pleospora herbarum* from red clover are a little longer than the measurements given by Saccardo (*17*) for that species. This fact gives some support to the possibility of considering the organism from red clover as *P. denotata*, a fungus which was described on dead stems of red clover and is separated from *P. herbarum* by the larger size of its ascospores. However, measurements of most of the spores of the red clover fungus come within the limits given for *P. herbarum* and it is considered best, for the present at least, to consider it as the latter species. Little doubt exists, however, that the organism described by Cooke and Ellis is the same as the one described here, but it is doubtful whether *P. denotata* can be considered as a species distinct from *P. herbarum*. Type material of *P. denotata* is very limited, but in the small portion examined the ascospores were found to be shorter than the length given in the original description of that fungus and most of them were within the limits given for *P. herbarum*. Many of the ascospores appeared as normal as ascospores from freshly collected field material, and it is assumed therefore that they had not become much shrunken. It seems better to extend the

limits of spore size of *P. herbarum* sufficiently to include the organism under consideration here rather than try to distinguish it as a distinct species on the difference of a few microns in spore length, particularly since the ascigerous and conidial stages correspond so closely to *P. herbarum* and its conidial stage, *Stemphylium botryosum*, respectively. Furthermore, it has been observed during these studies that environment has some effect on the size of ascospores of this fungus. Ascospores from perithecia on dead stems of red clover collected in the field measured 32μ to 48μ by 14μ to 21μ, whereas ascospores of the same fungus produced in culture on sweetclover stems measured 32μ to 54μ by 12μ to 22μ. However, the average size of the spores from red clover stems and sweetclover stems was 39.7μ by 17μ and 40.6μ by 16.8μ, respectively, from which it is apparent that the larger spores produced on sweetclover stems were comparatively few. This difference shows, however, that culture media and environmental conditions influence the size of ascospores to some degree.

From diseased leaves of alfalfa the writer isolated an echinulate-spored fungus that is considered to be *Stemphylium botryosum*. The pathogenicity of the fungus has been proved by inoculating alfalfa plants and again reisolating the fungus from the lesions produced. Conidia are rarely produced in culture, but abundant conidiophores and conidia are produced on leaf lesions. The conidiophores are sometimes branched, have the characteristic enlarged tips, and bear sarcinaeform spores that measure 24μ to 38μ by 14μ to 22μ. On potato-dextrose agar, the mycelium is white during the early stages of growth but later develops dark pigments that appear green from the top of the culture and dark brown or black from the bottom. On sweetclover stems or potato-dextrose agar, cultures of the organism produced *Pleospora herbarum* as the ascigerous stage. The ascigerous stage has not been observed on alfalfa stems collected in the field, but ascospores produced on sweetclover stems measured 32μ to 46μ by 13μ to 21μ.

Under field conditions alfalfa leaf lesions appear as dark-brown sunken areas that are often surrounded by a straw-colored halo (fig. 1, C and *D*). Concentric zones sometimes develop in old lesions.

Inoculation experiments indicate, however, that physiologic races of *Pleospora herbarum* exist on red clover and alfalfa. A suspension of conidia obtained from diseased alfalfa leaves was used to inoculate red clover and alfalfa plants. Leaves on alfalfa plants were completely killed in about 5 to 8 days, whereas no macroscopically visible infections appeared on red clover until 16 days after inoculation and then infection was very slight. When the two hosts were inoculated with a suspension of conidia from red clover, leaves of the latter host were killed in about 5 to 7 days after inoculation, whereas only small lesions were produced on alfalfa 14 days after inoculation. Similar inoculations were made with a suspension of ascospores and perithecial fragments from crushed perithecia, and in each case the organism collected from red clover was much more virulent on red clover than on alfalfa, while the organism collected from alfalfa was always more virulent on alfalfa than on red clover; in fact, the alfalfa organism rarely produced infection on red clover.

That the two organisms are different physiologic races is also supported by field observations. During the two seasons the red clover organism has been under observation the disease has appeared during

the early part of June, whereas in the season of 1938 the disease on alfalfa appeared in August and September. In June 1938, the disease was quite prevalent on red clover plants adjacent to a plot of alfalfa to which there was ample opportunity for the organism to spread. Several attempts were made to find it on the alfalfa but without success. While these data are not extensive enough to be conclusive, they do give evidence of some degree of physiological specialization on the two hosts.

Tehon and Daniels (*19*) described a brown leaf spot of alfalfa caused by an echinulate-spored fungus which they considered identical with *Macrosporium sarcinaeforme* Cav. Following Elliott's (*3*) suggestion that *M. sarcinaeforme* is not a good member of the genus *Macrosporium*, and assuming the forms on red clover and alfalfa to be identical, they erected a new genus, *Thyrospora*, with *T. sarcinaeforme* (Cav.) Tehon and Daniels, as the type species. It has already been shown by other investigators (*6*, *24*) and confirmed by the writer's own observations that Tehon and Daniels were incorrect in identifying their organism as *M. sarcinaeforme*, as the spores of the organism they described are echinulate, whereas spores of the red clover organism described by Cavara are smooth.

The organism described by Tehon and Daniels is here considered morphologically identical with *Stemphylium botryosum* described above on alfalfa. Spores and conidiophores obtained from diseased alfalfa specimens received from L. R. Tehon (fig. 4, *C*) correspond very well with spores and conidiophores of *S. botryosum* collected from diseased alfalfa by the writer (fig. 4, *B*). Wiltshire has already shown, moreover, that the genus *Thyrospora*, erected by Tehon and Daniels for this fungus, is identical with the genus *Stemphylium*, and he has accordingly transferred members of the genus *Thyrospora* to the genus *Stemphylium*.

Macrosporium medicaginis Cugini (*20*) apparently causes a disease of alfalfa similar to that caused by *Stemphylium botryosum*. It was hoped that a specimen of Cugini's collection could be obtained in order that the fungus described by him might be compared with *S. botryosum*. Since the organism was collected and described in Italy, an attempt was made to locate a specimen[4] at several stations in Italy, including the Experimental Agrarian Station at Modena, where Cugini worked. Since none was found it appears unlikely that any of the original material of *M. medicaginis* Cugini exists. Illustrations of leaf lesions given by Traverso (*20*) appear to have been drawn from lesions similar to those produced on alfalfa by *S. botroysum*. Spore measurements of *M. medicaginis* and *S. botryosum* are quite similar, but spore shape of *M. medicaginis*, as illustrated in Traverso's article, does not agree well with spore shape of *S. botryosum*. Furthermore the spores of *S. botryosum* are echinulate, whereas those of *M. medicaginis* are described as smooth. If this description is correct, then *M. medicaginis* is distinct from *S. botryosum*. In fact, the description given for *M. medicaginis* is not completely consistent with that of any fungus subsequently described on *Medicago sativa*. The fact that *Macrosporium medicaginis* is reported as approaching *M. meliloti*, a similarity that must have referred to shape since size

[4] For his efforts in trying to locate a specimen the writer is greatly indebted to Gabriele Goidanich, of the Vegetable Pathology Station at Rome, Italy.

is quite different, makes it unlikely that this is a report of *S. sarcinaeforme* attacking *Medicago sativa*.

An echinulate-spored fungus has been collected on alfalfa by several investigators in this country and in each case the fungus corresponds with *Stemphylium botryosum*, particularly as regards the echinulate character of the spores. Krakover states (*9, p. 279*), regarding material he examined from collections belonging to the herbarium of the United States Department of Agriculture,

> Slide mounts of spores on material collected in Philadelphia, Pa., and Arlington Farm, Va. (Turkestan alfalfa) contained spores which in shape and color are the same as the spores of *Macrosporium sarcinaeforme* on clover, but they are smaller in size and decidedly warty.

The writer has examined spore mounts of material furnished by F. R. Jones, which was collected on alfalfa in 1915 at Brookings, S. Dak., Geneva, N. Y., Ithaca, N. Y., and Mercer, Maine. In each case the spores were definitely echinulate. Thus it appears that *Stemphylium botryosum* has been collected on alfalfa several times and is probably more prevalent on that host than on red clover.

Malkoff (*10*) and Gentner (*5*) described a disease of red clover and alfalfa, and in each case they considered *Macrosporium sarcinaeforme* the etiological factor. Gentner obtained the perfect stage of the organism in culture and also collected it from old stems in the field. He identified it as *Pleospora herbarum*. Hülsenberg (*7*) reported a disease on red clover and alfalfa and considered it identical with the one described by Gentner and therefore attributed its cause to *M. sarcinaeforme*. In each of these cases, however, it was reported that the conidia of the causal fungus were echinulate. Since the conidia of *Stemphylium sarcinaeforme* (=*M. sarcinaeforme*) are devoid of warts or spines, it is evident that these investigators were not dealing with that fungus.

In correspondence with Gentner, the writer was advised to write to L. C. Doyer, of Wageningen, Netherlands, for seed infected with *Pleospora herbarum* or *Macrosporium sarcinaeforme*. From diseased seeds of red clover and alfalfa received from Doyer, ascospore cultures of an organism were obtained that appear identical with the organism described by Gentner (*5*). Cultures of the fungus grown on sweetclover stems at a temperature of 10° to 12° C. produced perithecia in about 10 to 14 days and ascospores in about 4 to 5 months. Except for a small difference in size, the measurements of the ascospores (32μ to 46μ by 14μ to 20μ) agree well with those of *Pleospora herbarum*, thus confirming Gentner's (*5*) opinion that the organism is that species. Very few conidia were produced on synthetic media, but abundant conidiophores and conidia were produced on leaf lesions. The conidia and conidiophore characters are very similar to those of *Stemphylium botryosum* described above on red clover and alfalfa, and the conidial stage is therefore considered to be that species. While conidia of the organism obtained from Doyer and conidia from the organisms collected from red clover and alfalfa show small differences in shape, these differences are not considered significant in view of the variation in shape of spores produced by each fungus (fig. 4, *A*, *B*, and *D*). In general the spores of the European culture are slenderer than those of the fungi collected by the writer. This difference is seemingly nullified, however, by the fact that practically as much

difference in spore shape occurs within a culture as between cultures. Gentner (*5*) observed considerable variation in size and form of spores collected from plants of different origin and pointed out that these characteristics are of little diagnostic value. Furthermore, when single-spore cultures from spores of either the slender type or the short thick type are used to inoculate plants, conidia of both types are produced on the leaf lesions by each type of isolate.

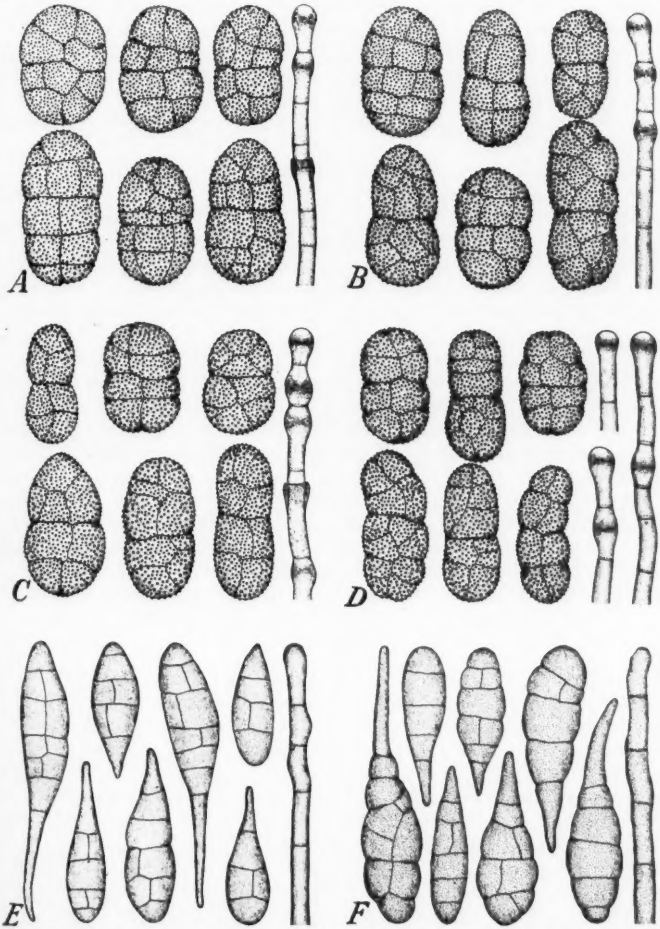

FIGURE 4.—Conidiophores and conidia: *A*, *Stemphylium botryosum* (conidial stage of *Pleospora herbarum*) collected from diseased red clover leaves; *B*, *S. botryosum* collected from diseased alfalfa leaves; *C*, *S. botryosum* from specimen of diseased alfalfa leaves, contributed by L. R. Tehon under the name *Thyrospora sarcinaeforme*; *D*, *S. botryosum* isolated from diseased red clover seeds obtained from L. C. Doyer; *E*, spores of a fungus described by Peck as *Macrosporium meliloti* on *Melilotus alba*; *F*, spores of *Macrosporium commune* from asparagus stems, collected by J. B. Ellis and deposited in the University of Wisconsin herbarium. × about 650.

Macrosporium meliloti Pk. (*14*) is described as occurring on living leaves of *Melilotus alba*. Since inoculations described earlier in this paper showed that *Stemphylium botryosum* is weakly pathogenic on sweetclover, it was thought that this might be the organism described by Peck. A small portion of the material collected by Peck was submitted to the writer by H. D. House. Spores obtained from the specimen are beaked (fig. 4, *E*) and therefore belong to the genus *Alternaria* as defined by Wiltshire (*23*), leaving no doubt that this fungus is different from those considered earlier in this paper. Spores of *Macrosporium meliloti* are very similar to those of *M. commune* Rabh. (fig. 4, *F*) collected by Ellis [5] on asparagus, and the two fungi probably belong in the same species of *Alternaria*.

Another organism that seems to warrant consideration here is *Pleospora lycopersici* É. and É. March. (*11*). This organism is reported (*11*) to be the perfect stage of *Macrosporium sarcinaeforme*, and in the figures that accompany the original description of the fungus the conidia are represented as smooth. This was probably an oversight, however, since Ramsey (*15*) isolated from tomatoes an organism that he considered the same as the one described by Marchal and Marchal, and the conidia are echinulate. Middleton (*12*) has recently shown by inoculation tests with *P. lycopersici* and *M. sarcinaeforme* on tomato and on a number of leguminous plants that the two organisms are distinct. Therefore *P. lycopersici* is not the perfect stage of the smooth, spored organism that occurs on red clover and it is doubtful that the perfect stage of this organism has as yet been found. The organism, described and illustrated by Ramsey, is very similar in both the conidial and perithecial stages to *P. herbarum* collected from red clover and alfalfa. A culture of *P. lycopersici* obtained from Ramsey was used to inoculate red clover and alfalfa by putting a small piece of agar and mycelium on the leaves and placing the plants in a glass-enclosed chamber for 4 or 5 days. The fungus proved slightly pathogenic on red clover but was less virulent than *P. herbarum* isolated from that host. No infection was obtained on alfalfa.

SUMMARY

Stemphylium botryosum Wallr. is reported herein as the cause of a foliage disease of red clover and alfalfa in Wisconsin.

The perfect stage of this fungus was found on dead red clover stems, and cultures from the typical echinulate conidia produced perithecia and ascospores when grown on sweetclover stems or potato-dextrose agar. The ascigerous stage is here identified as *Pleospora herbarum*.

The taxonomic relationship of this fungus to other similar fungi reported on these host plants is discussed.

Evidence is presented which indicates that physiologic races of the fungus exist on red clover and alfalfa.

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THE UTILIZATION BY CALVES OF ENERGY IN RATIONS CONTAINING DIFFERENT PERCENTAGES OF PROTEIN AND IN GLUCOSE SUPPLEMENTS [1]

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INTRODUCTION

A most important problem in the utilization of food energy by animals relates to the effect of combining individual feeds into rations. When rations are thus compounded, the value of the mixture in satisfying the energy requirements of an animal may not be the weighted mean of the values of the individual feeds. This is true, in the first place, because of the associative effects of feeds in digestion. The existence of these effects (but not their causes) has been amply established. In the second place, the supplementing effects of nutrients in metabolism may enhance the utilization of food energy. The realization of this latter fact is a relatively recent accomplishment in the science of nutrition. A historical description of its development has been given elsewhere (*25*).[3]

The effect of a variable intake of protein upon the utilization of food energy during growth was clearly stated by Møllgaard in 1929 (*29*) and demonstrated by the same investigator in earlier experiments on dairy cows. Visco (*34*) in 1930 showed that adult rats on protein-containing diets gained in body weight on energy intakes even smaller than those that, when provided by diets deficient in protein, could not maintain body weight. Hogan (*15*, *17*) and Forbes (*7*, *10*, *11*) and their associates have shown in various publications that, on equal intakes of food, growing rats will make greater gains and, in the Pennsylvania experiments (*7*, *10*, *11*), store more energy on rations containing moderate concentrations of protein (20 to 25 percent) than on rations containing low and obviously inadequate concentrations. Hamilton (*13*) confirmed these observations and in addition (*14*) proved that, for growing rats, the net availability of the metabolizable energy consumed increased as the percentage of protein (whole egg) in the diet increased up to about 16 percent, remained practically constant throughout the range of protein concentrations within which the diets were equally well-balanced, and then decreased markedly as the dietary protein was increased above 30 percent. These experiments of Hamilton support the general proposition that the better balanced a diet is with reference to a particular combination of animal functions, the smaller will be its energy wastage, represented in the heat increment, and the greater will be its net-energy value, digestive wastages remaining the same.

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[2] The authors wish to acknowledge gratefully the assistance in these experiments of Dr. F. I. Nakamura in the analysis of samples of chamber air.

[3] Italic numbers in parentheses refer to Literature Cited, p. 862.

The purpose of the experiments reported in this paper was to extend to beef calves the study of the relationship between the protein content of rations and the utilization of their energy content that had previously been carried out only with rats and dairy cows in milk. Also, since glucose supplements were used to dilute the protein content of the various basal rations used, an opportunity existed to determine the effect of this sugar on energy metabolism and to compute its net-energy value in the conventional way when incorporated in rations of diverse content of protein.

EXPERIMENTAL PLAN AND METHODS

The plan of the experiment involved the testing of 4 experimental rations, consisting essentially of combinations of corn, cottonseed meal, and timothy hay to provide roughly 6, 10, 15, and 20 percent of crude protein. Each of these rations was fed to each of 4 steer calves at a level to produce small positive energy balances, and immediately after such tests each basal ration was fed with a glucose supplement. The metabolizable energy in each ration (both basal and supplemented) was determined during a period of constant feed lasting from 13 to 28 days, and the heat production was determined in a 3-day period in the respiration chamber. In addition, the fasting heat production was measured for each steer at 5 different times during the experiment. Thus, the entire experiment included 32 collection periods for the determination of metabolizable energy and 52 respiration tests, the combination of which permitted the calculation of net energy.

The subjects of the experiment were four Hereford calves weighing initially from 400 to 500 pounds. They were confined in air-conditioned metabolism stalls, previously described (*16*, *26*), and were fed twice a day, at 9:30 a. m. and 4:30 p. m. They were weighed and watered each morning before feeding and watered again before the second feeding. The heat production was measured in an open-circuit type respiration chamber, also described in an earlier publication (*27*). In the respiration tests on feed, the steers were fed at 8 a. m., immediately after the beginning of the experimental day, and at 4 p. m., water being before them at all times. The tests on feed lasted 3 consecutive days. All fasting experiments were carried out on the fourth and fifth days of fast.

TABLE 1.—*Composition of the four experimental basal rations* [1]

Constituents	Ration containing—			
	6 percent [2] of crude protein	10 percent of crude protein	15 percent of crude protein	20 percent of crude protein
	Percent	*Percent*	*Percent*	*Percent*
Timothy hay	76.4	47.3	39.2	30.0
Ground corn	23.6	47.3	39.2	30.0
Cottonseed meal	0.0	5.4	21.6	40.0

[1] Hereafter called rations 6, 10, 15, and 20.

[2] For steer 1, the percentages were 92.3 and 7.7, respectively, for timothy hay and ground corn.

The composition of the four basal rations is given in table 1. Each basal ration was supplemented with glucose to produce rations that will be referred to as rations 6s, 10s, 15s, and 20s. In the last three of these supplemented rations each steer received daily 1,200 gm. (1,093 gm. of dry matter) of sugar in addition to the same amount of the corresponding basal ration as was consumed in the preceding test, except for ration 10s for steer 2, which included only 1,000 gm. of glucose. Because of its unpalatability, ration 6 was not readily consumed and could not be supplemented with as much glucose as the rations containing larger percentages of protein. Ration 6s for steers 1, 3, and 4, therefore, included only 400 gm. of glucose and for steer 2, 1,000 gm. While on each ration, each steer received daily 28 gm. of sodium chloride, 56 gm. of tricalcium phosphate, 30 gm. of dried yeast, and 10 gm. of a fortified cod-liver oil. The average protein content of the various rations, all supplements included, is given in table 2. The average composition and gross-energy content of the timothy hay, corn, cottonseed meal, and glucose used throughout the experiment are given in table 3.

In order to avoid possible age effects on the utilization of energy, the rations were fed in different order to the various steers, as indicated in table 4, which also gives the average body weights of the steers during both the collection periods and the respiration tests, which in most cases followed the corresponding collection periods. Each experimental feeding period was preceded by a preliminary period on the same amount of feed, varying in length from 5 to 18 days, with three exceptions when the preliminary feeding lasted only 3 days, i. e., for steer 2 on ration 15 and steer 3 on rations 10s and 20s. The dry matter and gross energy consumed daily in the various experimental periods are shown in table 5.

TABLE 2.—*Average protein content of dry matter of basal and supplemented rations*

Fed—	Rations 6 [1] and 6s	Rations 10 and 10s	Rations 15 and 15s	Rations 20 and 20s
	Percent	*Percent*	*Percent*	*Percent*
Unsupplemented	7.53	9.94	15.57	22.41
Supplemented with glucose	7.13	7.69	11.85	16.20

[1] For steer 1, the protein content of the unsupplemented ration was 6.77 percent.

TABLE 3.—*Average percentage composition and gross-energy content of feeds*

Feed	Dry matter	Crude protein	Crude fiber	Gross energy per gram
	Percent	*Percent*	*Percent*	*Calories*
Timothy hay	91.41	5.53	33.46	4.052
Ground corn	88.79	9.21	2.01	3.985
Cottonseed meal	92.90	40.31	10.93	4.557
Glucose	91.09			3.322

TABLE 4.—*Order of feeding experimental rations and average body weights of steers on different rations*

Steer No.	Ration	Order of feeding	Average body weight of steer during—		Ration	Order of feeding	Average body weight of steer during—		Ration	Order of feeding	Average body weight of steer during—		Ration	Order of feeding	Average body weight of steer during—	
			Collection period	Respiration period			Collection period	Respiration period			Collection period	Respiration period			Collection period	Respiration period
			Kilograms	*Kilograms*			*Kilograms*	*Kilograms*			*Kilograms*	*Kilograms*			*Kilograms*	*Kilograms*
1	6	First	211	204	6s	Second	218	219	10	Third	208	211	10s	Fourth	225	230
2	6	Third	228	224	6s	Fourth		232	10	First	205	209	10s	Second	229	224
3	6	Fifth	285	291	6s	Sixth	301	304	10	Third	259	261	10s	Fourth	274	278
4	6	Seventh	316	315	6s	Eighth	327	333	10	Fifth	297	297	10s	Sixth	313	309
1	15	Fifth	228	233	15s	Sixth	254	159	20	Seventh	254	260	20s	Eighth	272	280
2	15	Seventh	266	271	15s	Eighth	290	296	20	Fifth	235	224	20s	Sixth	264	259
3	15	First	216	223	15s	Second	251	251	20	Seventh	297	305	20s	Eighth	323	335
4	15	Third	269	264	15s	Fourth	289	286	20	First	237	239	20s	Second	263	269

TABLE 5.—*The dry matter and gross energy consumed daily in the experimental periods when steers were on different rations*

Steer No.	Ration	Dry matter consumed daily	Gross energy consumed daily	Ration	Dry matter consumed daily	Gross energy consumed daily	Ration	Dry matter consumed daily	Gross energy consumed daily	Ration	Dry matter consumed daily	Gross energy consumed daily
		Kilograms	*Calories*		*Kilograms*	*Calories*		*Kilograms*	*Calories*		*Kilograms*	*Calories*
1	6	2.946	12,835	6s	2.899	12,424	10	2.807	12,399	10s	3.869	16,240
2	6	3.232	14,191	6s			10	2.721	12,108	10s	3.751	15,811
3	6	3.954	17,377	6s	4.364	18,551	10	3.152	13,937	10s	4.237	17,880
4	6	4.097	17,959	6s	4.504	19,383	10	3.617	16,063	10s	4.732	19,833
Average		3.557	15,590		3.922	16,786		3.074	13,627		4.147	17,441
1	15	3.139	14,159	15s	4.245	17,949	20	3.002	13,701	20s	4.119	17,743
2	15	3.331	14,952	15s	4.455	19,020	20	2.868	12,910	20s	3.961	16,932
3	15	2.797	12,681	15s	3.899	16,559	20	3.110	14,188	20s	4.228	18,246
4	15	3.186	14,362	15s	4.275	18,292	20	2.736	12,512	20s	3.913	16,840
Average		3.113	14,038		4.218	17,955		2.929	13,328		4.055	17,440

The fasting periods were interposed among the various feeding periods in such a way as to secure information concerning the effects of high and low levels of prefeeding on the heat production during the fourth and fifth days of a following fast. One fasting period preceded all feeding periods for each steer and two fasting periods were run after the regular schedule of feeding, one immediately after the last experimental period, which for all steers was a period in which a sugar-supplemented ration was fed, and the other following an adequate feeding period of at least 2 weeks on the same ration minus its sugar supplement. The other fasting periods were, for all steers, interposed between the second and third and the sixth and seventh feeding periods.

All rations during each period and all collections of urine and feces were analyzed for nitrogen and their heats of combustion (gross energy) determined in an adiabatic Parr oxygen-bomb calorimeter. A few were analyzed also for crude fiber by the official method of the Association of Official Agricultural Chemists.

All calorimetric calculations were made on the basis of daily heat productions corrected to a standard day of 12 hours standing and 12 hours lying down, following the procedure introduced by Armsby.

TABLE 6.—*Fasting heat production of the steers expressed in calories per day per* $W^{.75}_{kg}$

Steer No.	Heat production for fasting period No.—					Average
	1	2	3	4	5	
	Calories	*Calories*	*Calories*	*Calories*	*Calories*	*Calories*
1	64.53	56.39	79.05	76.15	64.42	68.11
2	71.39	74.92	78.03	81.24	71.76	75.47
3	72.99	81.87	70.17	71.99	69.19	73.24
4	70.34	78.45	69.59	67.18	65.29	70.17
Average	69.81	72.91	74.21	74.14	67.67	71.75

EXPERIMENTAL RESULTS

FASTING HEAT PRODUCTION

The fasting heat production (table 6) varied from period to period in a disturbing and unexplainable fashion, an experience that Forbes and his associates also recorded (*8*). The values showed no correlation with the average respiratory quotients prevailing during the test nor with the level of feeding immediately preceding the test. The coefficient expressing the correlation between the fasting heat production per day and the daily dry matter consumed in the immediately preceding period, both expressed to the body weight in kilograms raised to the 0.75 power, was +0.188 for the 20 determinations. According to Fisher (*5, p. 176*), for a probability no smaller than 0.1 that the indicated correlation is a result of the operation of fortuitous factors only, the coefficient should be as large as 0.36. It may reasonably be concluded, therefore, either that no correlation exists or that it is quite inappreciable in comparison with other determining factors.

For the purpose of computing the heat increments of the basal rations, the average heat production for each steer per $W^{.75}_{kg.}$ was used, as given in the last column of table 6. The variations in fasting heat production were thus not considered in the calculations simply because

the heat increments obtained by so doing were considerably more variable than those obtained on the assumption of a constant basal metabolism.

The day's heat production dropped from the fourth to the fifth day of fast in 16 of the 20 tests, but on an average this decrease amounted to only 3.44 percent, possibly of the same order of magnitude as the decrease in body weight. The average respiratory quotient for the fourth day of fast was 0.727, and for the fifth day, 0.739.

HEAT INCREMENT OF GLUCOSE AFTER FASTING

Following the second fasting period for each steer, glucose was given as follows, and the heat production determined for the subsequent 12 hours, or, in one case, 24 hours: Steer 1, 100 gm. at 8 a. m., 12 m., and 4 p. m.; steer 2, 500 gm. at 8 a. m. and 2 p. m., though of the latter only 65 gm. was consumed; steer 3, 500 gm. at 8 a. m.; steer 4, 500 gm. at 8 a. m. For steer 3, sugar feeding was repeated after the third fasting period, 600 gm. being given at 8 a. m. The respiration results pertaining to these sugar tests are summarized in table 7.

TABLE 7.—*Effect of glucose feeding on the fasting metabolism of steers*

Steer No.	Heat production during fast [1]		Glucose given	Heat production [2] after sugar feeding	Average respiratory quotients		
					Fasting		After sugar feeding
	Fourth day	Fifth day			Fourth day	Fifth day	
	Calories	*Calories*	*Grams*	*Calories*			
1	3,101	2,916	300	1,544	0.815	0.857	0.912
2	4,187	4,297	565	2,335	.757	.716	.800
3	5,133	5,029	500	2,508	.711	.712	.781
3	4,947	4,845	600	[1] 4,921	.745	.756	.790
4	5,067	5,040	500	2,492	.736	.705	.792

[1] 24-hour period.
[2] 12-hour period only except for second value for steer 3.

The heat production of the steers was practically unaffected by sugar feeding, or inappreciably affected, except in the case of steer 2, although the respiratory quotients indicated sugar oxidation in all cases. The percentage differences between the heat production (expressed per 24 hours) following sugar feeding and the heat production of the fifth day of fast were +5.7 for steer 1, +8.3 for steer 2, −0.26 and +1.56 for steer 3, and −1.12 for steer 4. The last three deviations are well below the expected (average) percentage deviation between successive days on the same feed (2.65, see p. 858), and the first deviation is not improbably beyond the expected range. If the deviation for steer 2 represents a true specific dynamic effect, its magnitude has been greatly exaggerated by the method of calculation, according to which the heat production of the 12-hour subperiod within which the sugar was given and undoubtedly most of the heating effect was exerted was multiplied by 2.

Tests for sugar in the urine following sugar feeding were negative. In the second test on steer 3, the heat production for the first 12 hours

was 2,561 calories and for the second 12 hours, 2,360 calories. The respiratory quotients in successive 6-hour periods following the consumption of 600 gm. of sugar were 0.764, 0.794, 0.858, and 0.744.

Evidently under the conditions of these experiments, glucose may exert no specific dynamic effect, even though it undergoes oxidation. That sugar oxidation was not more pronounced than the rather small rises in the respiratory quotient indicate may be because "it is more important to replenish the carbohydrate stores than to use the incoming carbohydrate for fuel" (*18*). Sugar feeding did not accelerate the paunch fermentation, since subsequent to glucose ingestion the chamber air contained little or no methane.

USE OF THE FASTING HEAT PRODUCTION IN THE COMPUTATION OF HEAT INCREMENTS OF FEED

The purpose of determining the fasting heat production of the experimental steers was to afford a base for the computation of the heat increments due to feeding. This is a commonly used procedure, both among physiologists (such as Rubner and Lusk) interested in the stimulating effect of nutrients on metabolism, and among nutritionists (such as Benedict) interested in determining the losses of food energy in metabolism as extra animal heat. Forbes and his associates (*9*) have used the same procedure in determining the utilization of feed energy for the maintenance of dairy cattle, but since 1928 these investigators have decided that this procedure is grossly in error. They now believe that the fasting heat production of an animal consists of two components, a hypothetical minimum basal level of metabolism and an increment representing the waste heat of the body nutrients catabolized during fast. On this understanding, the difference in heat production between the fasting condition and any level of feeding does not give the true heat increment of the food but gives, rather, this increment minus the waste heat saved by the sparing of body nutrients. Originally proposed as a theory to account for the departure from linearity below the point of energy equilibrium of the curve relating heat production in cattle to the intake of dry matter, this understanding of the nature of the fasting catabolism has assumed the character of an established fact in the minds of its proponents, on the basis of which the work of others has been severely criticized (*6*, *11*, *22*).

It is the intention of the writers to use the classical method of computing heat increments in this paper in estimating the net energy values of the experimental rations. The arguments that have been advanced in favor of the theory of Forbes (*20*) are unconvincing, mainly because the supporting data cited are open to other interpretations than those selected. In other words, the supporting evidence may be considered as in harmony with the theory, but not as demonstrating its truth. The experimental evidence adduced by Kriss, Forbes, and Miller (*22*) is open to criticism on the basis both of the experimental technic used and of the methods of correcting the heat increments of dietary nutrients for the assumed waste energy of body nutrients spared. And finally it may be said that some experimental evidence may be cited that is difficult, if not impossible, to harmonize with the theory.

TABLE 8.—*Nitrogen and energy balances per day with steers on different rations*

Steer No.	Ration	Nitrogen balance	Energy balance	Ration	Nitrogen balance	Energy balance	Ration	Nitrogen balance	Energy balance	Ration	Nitrogen balance	Energy balance
		Grams	*Calories*		*Grams*	*Calories*		*Grams*	*Calories*		*Grams*	*Calories*
1	6	+4.79	−675	6s	(−1.66)		10	+13.28	+1,090	10s	+3.60	+2,162
2	6	+8.61	+444	6s			10	+12.15	−173	10s	+8.06	+779
3	6	+4.30	+2,798	6s	+6.92	+2,470	10	+6.85	+492	10s	+14.69	+1,405
4	6	+4.91	+1,052	6s	+3.86	+1,798	10	+9.25	+1,838	10s	+8.88	+2,805
Average		+5.65	+905		+5.39	+2,134		+10.38	+812		+8.81	+1,788
1	15	+19.22	+1,774	15s	+19.18	+1,916	20	+13.78	+1,247	20s	+20.85	+2,262
2	15	+15.62	+1,727	15s	+21.65	+1,923	20	+29.42	+1,231	20s	+29.11	+2,356
3	15	+17.88	+836	15s	+23.08	+1,793	20	+19.55	+1,414	20s	+23.95	+2,625
4	15	+9.36	+1,134	15s	+23.32	+3,175	20	+16.70	+987	20s	+18.96	+2,631
Average		+15.52	+1,368		+21.81	+2,202		+19.86	+1,220		+23.22	+2,469

BALANCES OF NITROGEN AND OF ENERGY

Although the attempt was made to feed the various basal rations at the maintenance level, this proved to be more difficult to attain in the time available for the tests than was anticipated, largely because the steers were still in the period of active growth. In the desire to avoid submaintenance feeding in particular, too much of the rations was fed in all cases but two, and positive energy balances were obtained ranging, on an average, from 812 to 1,368 calories per day, and in individual cases from 492 to 2,798 calories per day (table 8), the next highest individual value being 1,838 calories. The results with the lowest protein levels, rations 6 and 6s, cannot, unfortunately, be considered satisfactory because of the unpalatability of the rations and the consequent irregularity in the consumption of food.

The average increases in energy balance brought about by the sugar supplements were 976, 834, and 1,249 calories per day for rations 10s over 10, 15s over 15, and 20s over 20, respectively. The individual differences were quite variable, especially between rations 15 and 15s.

The nitrogen balances increased with increasing percentages of protein from an average of 5.65 to 19.86 gm. per day. The protein-sparing effect of the sugar supplements was evident only with rations 15s and 20s, their nitrogen balances being quite definitely greater than those of the corresponding basal ration.

TABLE 9.—*Losses of chemical energy, and metabolizable energy of different rations fed to steers*

Ration and steer No.	Energy per kilogram of dry matter					Percentage energy			
	Total	Lost in—			Metabolizable	Lost in—			Metabolizable
		Feces	Urine	Methane		Feces	Urine	Methane	
Ration 6:	*Calories*	*Calories*	*Calories*	*Calories*		*Percent*	*Percent*	*Percent*	*Percent*
1	4,357	2,093	138	341	1,784	48.05	3.17	7.82	40.96
2	4,391	1,951	107	354	1,979	44.43	2.43	8.06	45.08
3	4,395	1,544	130	269	2,451	35.14	2.95	6.13	55.78
4	4,383	1,653	130	337	2,264	37.71	2.96	7.68	51.65
Average [1]	4,390	1,716	122	320	2,231	39.09	2.78	7.29	50.84
Ration 6s:									
1	4,286	2,126	77.3	341	1,742	49.61	1.80	7.95	40.64
2									
3	4,251	1,505	113	283	2,349	35.42	2.66	6.67	55.25
4	4,304	1,680	113	335	2,176	39.03	2.63	7.78	50.56
Average	4,280	1,770	101	320	2,089	41.35	2.36	7.47	48.82
Ration 10:									
1	4,417	1,643	84.4	375	2,314	37.20	1.91	8.50	52.39
2	4,450	1,812	92.6	266	2,279	40.72	2.08	5.98	51.22
3	4,422	1,781	91.1	345	2,205	40.28	2.06	7.80	49.86
4	4,441	1,459	106	272	2,604	32.85	2.38	6.13	58.63
Average	4,432	1,674	93.5	314	2,350	37.76	2.11	7.10	53.03
Ration 10s:									
1	4,171	1,356	76.3	211	2,527	32.52	1.83	5.06	60.59
2	4,215	1,854	57.1	206	2,099	43.98	1.35	4.88	49.79
3	4,220	1,649	68.7	297	2,206	39.07	1.63	7.04	52.26
4	4,191	1,473	73.5	303	2,342	35.14	1.75	7.22	55.89
Average	4,199	1,583	68.9	254	2,294	37.68	1.64	6.05	54.63

[1] Omitting the data for steer 1.

TABLE 9.—*Losses of chemical energy, and metabolizable energy of different rations fed to steers*—Continued

Ration and steer No.	Energy per kilogram of dry matter					Percentage energy			
	Total	Lost in—			Metabolizable	Lost in—			Metabolizable
		Feces	Urine	Methane		Feces	Urine	Methane	
Ration 15:	*Calories*	*Calories*	*Calories*	*Calories*		*Percent*	*Percent*	*Percent*	*Percent*
1	4,511	1,374	119	317	2,700	30.47	2.65	7.03	59.85
2	4,489	1,303	114	318	2,754	29.02	2.54	7.09	61.35
3	4,534	1,539	126	283	2,586	33.95	2.78	6.24	57.03
4	4,508	1,641	106	345	2,416	36.40	2.35	7.65	53.60
Average	4,510	1,464	116	316	2,614	32.46	2.58	7.00	57.96
Ration 15s:									
1	4,228	1,223	88.6	293	2,624	28.93	2.09	6.93	62.05
2	4,269	1,260	78.8	348	2,582	29.52	1.85	8.16	60.48
3	4,247	1,346	62.8	241	2,597	31.70	1.48	5.68	61.14
4	4,279	1,270	68.5	322	2,619	29.67	1.60	7.52	61.21
Average	4,256	1,275	7.47	301	2,606	29.96	1.76	7.07	61.21
Ration 20:									
1	4,564	1,319	139	332	2,774	28.90	3.04	7.28	60.78
2	4,501	1,310	122	313	2,757	29.09	2.71	6.96	61.24
3	4,562	1,297	148	349	2,768	28.44	3.24	7.65	60.67
4	4,573	1,380	137	277	2,779	30.17	3.00	6.06	60.77
Average	4,550	1,326	136	318	2,770	29.15	3.00	6.99	60.86
Ration 20s:									
1	4,308	1,126	83.8	396	2,701	26.15	1.94	9.20	62.71
2	4,275	1,266	52.8	228	2,728	29.61	1.23	5.33	63.83
3	4,316	1,123	74.5	329	2,790	26.01	1.73	7.62	64.64
4	4,304	1,360	75.6	260	2,608	31.60	1.76	6.05	60.59
Average	4,301	1,219	71.7	303	2,707	28.34	1.66	7.05	62.95

METABOLIZABLE ENERGY IN THE RATIONS

The wastages of energy in urine, feces, and methane,[4] and the matabolizable energy remaining for the various rations and steers are summarized in table 9. The following points are noteworthy:

The metabolizability of the gross energy of the basal rations increased progressively from ration 6 to ration 20 as the following average percentages show: 50.84, 53.03, 57.96, and 60.86. This is probably not an effect of the increasing protein percentage but a result of the increasing proportion of concentrates and the decreasing proportion of timothy hay.

Although glucose itself is completely absorbable and highly metabolizable, the metabolizable energy as a percentage of the gross energy in the basal rations suplemented with glucose to the extent of about 30 percent of their gross energy is only slightly greater than that of the basal rations alone. These slight differences are in part the result of smaller proportionate losses of energy in the urine, occasioned by a reduction in the proportion of protein in the rations, and in part the result of variable and inconsiderable decreases in the proportionate loss of energy in the feces. It is noteworthy that neither the absolute amounts of methane produced nor the percentage loss of energy in methane was appreciably different for the rations supple-

[4] The methane was determined in the chamber air by analysis, using the gasometric methane analyzer of Carpenter.

mented with glucose as compared with the basal rations. Obviously the glucose supplements, though highly fermentable themselves, did not stimulate fermentation in the paunch insofar as methane production is a criterion.

TABLE 10.—*Net-energy values of rations*

Steer No.	Net energy value											
	Ration	Per kilogram of dry matter	As percentage of metabolizable energy	Ration	Per kilogram of dry matter	As percentage of metabolizable energy	Ration	Per kilogram of dry matter	As percentage of metabolizable energy	Ration	Per kilogram of dry matter	As percentage of metabolizable energy
		Calories	*Percent*		*Calories*	*Percent*		*Calories*	*Percent*		*Calories*	*Percent*
1	6	[1] 1,019	[1] 57.1	6s	.....	.....	10	1,732	74.9	10s	1,597	62.8
2	6	1,489	75.2	6s	.....	.....	10	1,463	64.2	10s	1,373	65.4
3	6	2,014	82.2	6s	1,787	76.1	10	1,664	75.5	10s	1,510	68.4
4	6	1,538	67.9	6s	1,614	74.2	10	1,898	72.9	10s	1,685	71.9
Average	.....	1,680	75.1	.....	1,701	75.1	.....	1,689	71.9	.....	1,541	67.1
1	15	1,859	68.9	15s	1,486	56.6	20	1,884	67.9	20s	1,682	62.3
2	15	2,032	73.8	15s	1,639	63.5	20	1,954	70.9	20s	1,826	66.9
3	15	1,813	70.1	15s	1,646	63.4	20	2,175	78.6	20s	1,978	70.9
4	15	1,800	74.5	15s	1,884	71.9	20	1,918	69.0	20s	1,865	71.5
Average	.....	1,876	71.8	.....	1,664	63.9	.....	1,983	71.6	.....	1,838	67.9

[1] Not included in the averages because of irregular consumption of food.

NET ENERGY OF THE RATIONS

The net-energy values of the rations, expressed both in calories per kilogram of dry matter and in percentage of the metabolizable energy, are given in table 10. These values have all been computed by the use of heat increments observed above the fasting heat production, estimated in each feeding period from the average body weight of the steer and the average for the steer of the results of the five basal metabolism periods, expressed in calories per $W_{kg}^{.75}$. In the discussion of table 10, the results for rations 6 and 6s will not be considered because of their incompleteness and of their impaired significance brought about by the irregular consumption of food.

Expressed on the dry-matter basis, the net-energy content of the three basal rations increased, on an average, with the increase in protein content, the values in order being 1,689, 1,876, and 1,983 calories per kilogram. However, the metabolizable energy on the same basis also increased—2,350, 2,614, and 2,770 calories (table 9)—so net availability of the metabolizable energy averaged practically the same for all, i. e., 71.9, 71.8, and 71.6 percent. Although the protein content of ration 10, and to a less extent of ration 15, was definitely inadequate for the maximum retention of nitrogen (see table 8), the insufficiency evidently did not impair the utilization of the metabolizable energy.

The growing beef steer thus differs from the dairy cow in milk and from the growing rat in that the metabolizable energy of rations inadequately balanced with respect to protein is just as well utilized as the metabolizable energy of rations containing adequate amounts of

protein. It is possible that in the improved beef breeds of cattle the process of fat formation is just as efficiently performed, at least within certain limits, as the process of growth with respect to the utilization of food energy. In such a case, food energy consumed above the quota of balanced nutrients in the ration that can be used in the synthesis of organic complexes for the production of new body tissue is diverted to the adipose tissues and there synthesized to fat with an efficiency equal to that realized in the growth process. This would mean that the heat increment of nutrients used in fattening is no greater than that of nutrients used in growth. If these heat increments are determined by the plethora of nutrient material in the intercellular fluids, and, for a given intake of food, if the plethora is dependent on the rate of withdrawal of the end products of digestion from these fluids, the equality of utilization of energy in fattening and in growth implies an equality in rates of synthesis of body fat and of protoplasm.

On the same basis of reasoning, Møllgaard's analogous work on dairy cows in milk (*28, pp. 160–180*) would mean that the rate of synthesis of the organic constituents of milk is greater than the rate of synthesis of body fat and of protoplasm, a proposition that is perfectly obvious.

On the other hand, it is not surprising that such an animal as the albino rat, which does not fatten readily, since it has not been selectively bred with that end in view, should construct protoplasm during growth at a faster rate than it can dispose of excess food by synthesis into fat. Hence, of two comparable rations, the one with a content of protein permitting the greater utilization of food energy in growth would produce the smaller heat increment. This Hamilton (*13*) has shown in his carefully controlled experiments.

The variability of the net-energy values for the individual steers and the 6 experimental rations was not extreme. The root-mean-square deviation of all 24 values from their respective means was 127 calories, equivalent to 7.20 percent of the mean net-energy value, i. e., 1,765 calories. As previously reported (*26*), the writers have computed from 4 recent publications of Forbes and his associates an average percentage difference of 7.1 between pairs of determinations on different steers of the net-energy value of rations for maintenance, with individual differences as high as 13.9 percent. The earlier work of Armsby shows individual differences up to 24 percent. Kleiber (*21*) has calculated a coefficient of variation of 11 percent exhibited by a series of 11 determinations of the net-energy value of starch reported by Kellner and Kühn.

The percentage differences between the heat productions during adjacent days in the calorimeter of the steers in this experiment while on feed averaged 2.65 ± 1.30 for 66 comparisons, as compared with an average of 2.73 ± 1.48 for 75 similar comparisons from the work of Armsby and Fries (*1*). The probable errors appended to the above means define a range within which the probability that a single deviation will fall is 0.5.

AVAILABILITY OF THE ENERGY OF GLUCOSE SUPPLEMENTS

By the usual methods of estimating the metabolizable and net energy of supplemental feeds added to a basal ration, the energy values of the glucose supplements to the various basal diets used in these experi-

ments have been determined, making allowances for differences in the body weights of the steers in comparable periods and for small differences in the consumption of the basal rations. The results of these estimations are summarized in table 11.

TABLE 11.—*Metabolizable and net-energy values of the glucose supplements in the different rations fed*

Rations from which computed and steer No.	Glucose calories in percentage of basal calories	Energy per kilogram of dry matter		Percentage net availability of metabolizable energy
		Metabolizable	Net	
Rations 10 and 10s:	*Percent*	*Calories*	*Calories*	*Percent*
1	32	3,131	1,250	40
2	26	1,536	1,094	71
3	28	2,209	1,066	48
4	25	1,471	978	66
Average	28	2,087	1,097	56
Rations 15 and 15s:				
1	29	2,402	410	17
2	25	2,054	431	21
3	31	2,624	1,219	46
4	28	3,210	1,895	59
Average	28	2,572	989	36
Rations 20 and 20s:				
1	29	2,500	1,117	45
2	31	2,654	1,488	56
3	28	2,853	1,416	50
4	31	2,166	1,737	80
Average	30	2,543	1,439	58

The great variability exhibited by the individual estimates, particularly the low net-energy values for steers 1 and 2 on ration 15s, are to be ascribed in part to the fact that the glucose supplements could not be fed in amounts greater than 1,200 gm. if consistently complete consumption was to result with all basal rations. This amount on the gross-energy basis was equal to only 30 percent of the basal ration. Indirect determinations of heat increments under such conditions cannot possess great accuracy. The variability may also be a result of the extreme irregularity of glucose absorption in the ruminant as revealed by the studies of Von Teichmann and Logischen (*33*) and of Krzywenek and Brüggemann (*23*) on the blood-sugar curve in cattle following glucose feeding. Since sugar is readily fermentable in the paunch, its destruction by the paunch organisms may be expected to vary with conditions not under the control of the investigator. Fraps (*12*) has shown, for example, from a study of digestion coefficients in American experiments published prior to 1924, that the digestibility of crude fiber in ruminants, brought about entirely by the paunch organisms, is two or three times as variable as the digestibility of protein- and nitrogen-free extract.

Because of this variability, average differences in the utilization of the energy of the glucose supplements for the different basal rations cannot be considered significant. The average of all 12 estimates indicates that glucose, with a gross energy value of 3,680 calories per kilogram of dry matter, possesses on the same basis 2,401 calories of

metabolizable energy and 1,175 calories of net energy. An average of 65 percent of its gross energy was metabolizable, while of the metabolizable energy 50 percent was net available.

As far as the writers are aware, calorimetric determinations of the value of glucose in cattle feeding have not been reported in the literature. However, comparison of the above values with those reported for starch is of interest. Armsby and Fries (*2*) have reported 1,636 calories per kilogram as the net-energy value of starch, 81.1 percent of the gross energy being metabolizable, and 49.2 percent of the latter being net available. The results of Kellner and Köhler (*20*), recomputed by Armsby and Fries (*2*), assign a value of 1,803 calories of net energy to the kilogram of starch, with 73.8 percent of the gross energy metabolizable and 58.8 percent of the latter net available. The values found for glucose in the writers' experiments are lower than those reported for starch, the difference being in the metabolizability of the gross energy, which was 65.2 percent for glucose and 81.1 (Armsby) and 73.8 (Kellner) for starch. The net availability of the metabolizable energy was not greatly different for glucose and starch, being 50 percent for the former and 49.2 (Armsby) and 58.8 (Kellner) for the latter.

Kellner (*19*) has reported a net-energy value for sucrose that is 24 percent less than that found for starch and has attributed this deficit to the ready fermentability of sucrose. In the writers' experiments, glucose did not increase the methane production over that of the basal rations (table 9). However, as Woodman and Evans (*36*) report, paunch bacteria are able to ferment glucose much more readily than cellulose, so that glucose would be expected to depress the fermentation of cellulose and other polysaccharides indigestible by body-enzyme action. This would be an associative effect of glucose in digestion that would depress the estimated metabolizable energy of glucose below its true value.

TABLE 12.—*Digestibility of crude fiber in basal rations and in rations supplemented with glucose*

Steer No.	Ration	Digestibility of crude fiber	Ration	Digestibility of crude fiber
		Percent		*Percent*
1	6	51.72	6s	36.15
2	10	36.65	10s	21.35
3	15	35.48	15s	29.70
4	20	35.90	20s	31.76
Average		39.94		29.74

To determine whether such an effect was manifested in this experiment, the digestibility of crude fiber was determined for one of the steers on each of the basal rations, unsupplemented and supplemented with glucose, with the results given in table 12. There was in fact a distinct depression in the digestibility of the crude fiber, amounting to more than 25 percent, and this depression would be experienced not only by the cellulose contained in the crude fiber but also by that present in the nitrogen-free extract (*30*) and probably by the hemi-

celluloses that are also dependent on bacterial action for their digestion.[5]

Hence, it seems fair to conclude that the low metabolizability of the gross energy of glucose by cattle, and presumably other ruminants, is a result both of its own partial destruction by the paunch flora and fauna and of its inhibition of the fermentation of the insoluble polysaccharides of the basal ration that depend upon such fermentation for their digestive utilization. A definite depressing effect of molasses on the digestibility of crude fiber in sheep, quite analogous to the effect of glucose demonstrated in table 12, has been reported by Briggs (*3*)—notwithstanding his conclusions to the contrary—and by Scharrer and Nebelsiek (*31*). No uniformly depressing effect of cane molasses on the digestibility of crude fiber by dairy cows was observed by Williams (*35*). The depressing effect of glucose on the digestion of the insoluble carbohydrates of feeds would not be produced in calves less than 3 months of age because the paunch is not formed until then, according to the X-ray studies of Magee (*24*).

However, the poor utilization of glucose cannot be traced entirely to its effect upon the digestive process in the paunch, since Fingerling and Schoenemann (*4*) and Schoenemann (*32*) have shown that another sugar, sucrose, giving rise to glucose in digestion, has a lower net-energy value than starch, not only for the steer but also for the pig and the dog, in which extensive destruction of carbohydrates by bacterial fermentation does not occur. Furthermore, the ratio of the net-energy value of sucrose to that of starch was not greatly different among these diverse species, being 1:1.37 for the dog, 1:1.28 for the pig, and 1:1.32 for the steer. The nature of this difference between the utilizations of starch and sucrose is unknown and is an interesting subject for future research.

CONCLUSIONS

In experiments upon four steer calves, involving 32 collection periods for the determination of metabolizable energy and 52 tests in the respiration chamber, results were obtained that support the following conclusions:

(1) The utilization of the metabolizable energy in the rations of growing calves is not impaired by inadequate levels of protein within the limits tested. Apparently, the utilization of food energy in fattening is as efficient as that in growth.

(2) Glucose given in moderate amounts to calves that have fasted for 5 days may exert no specific dynamic effect, even though the respiratory quotient indicates its combustion. It does not depress the fasting level of heat production, even though it probably spares body protein. This fact seems incompatible with the theory that the body nutrients catabolized during fasting exert a considerable specific dynamic effect.

(3) The addition of glucose to a basal ration depresses the digestibility of those insoluble carbohydrates (celluloses, hemicelluloses) that depend on the fermentative action of the micro-organisms of the paunch for their digestion. At the same time, the extent of these fermentations, as measured by the production of methane, is not

[5] This marked depression in the digestibility of crude fiber in ruminant animals by the ingestion of glucose has been confirmed by similar (unpublished) experiments on sheep.

increased. This effect is apparently a result of the substitution of glucose for insoluble carbohydrates as the substrate of the bacteria of the paunch.

(4) Because of this associative effect between glucose and the insoluble carbohydrates of the basal ration, estimations of the metabolizability of the gross energy of glucose supplements, averaging 65 percent, are much lower than the true values.

(5) Under the conditions of feeding followed in this experiment, about 50 percent of the metabolizable energy of glucose supplements to a basal ration is wasted in the increment in heat production thus induced. This wastage is comparable to that reported from other laboratories for starch and sucrose.

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TWO TYPES OF FALL PANICUM SMUT[1]

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INTRODUCTION

The common smuts are characterized by black or brown, dusty masses of chlamydospores that break out on the surface of the infected plants. A few cases have been recorded, however, in which the sori of the common smuts have been composed of hyaline chlamydospores and hence were light-colored instead of dark. Campagna,[2] in 1926, reported the finding of a single smutted head of wheat at Ste. Anne de la Pocatiere, Quebec, Canada, which morphologically showed all the symptoms of the ordinary loose smut (*Ustilago tritici* (Pers.) Rostr.), but differed from it by the fact that the spores were hyaline and the spore mass was white instead of black. His attempts to germinate the spores all failed and no artificial inoculations were tried, so that no conclusions could be reached as to the pathogenicity and stability of the organism. He suggested that the white smut was perhaps a case of mutation or sudden variation.

Holton,[3] in 1931, reported a buff type of oat smut in which the chlamydospores were smooth and colorless, in contrast to the dark-brown chlamydospores of the common type of oat smut. This "buff" smut was produced by a cross between two monosporidial lines that originated from interspecific hybrid chlamydospores (*Ustilago avenae* (Pers.) Jens. × *U. levis* (Kell. and Sw.) Magn.). Further studies led Holton [4] to conclude that the buff smut was a result of mutation in *U. levis* and that the change involved only the factor that determines color in the chlamydospores.

Moore and Allison,[5] in 1935, reported the finding of a single smutted head of barley at St. Paul, Minn., infected with what appeared to be an albino strain of *Ustilago hordei* (Pers.) Kell. and Sw. The head was almost white and was intermediate between the loose smut and the covered smut types. The spores were hyaline, smooth, and somewhat smaller than those of normal *U. hordei*. They produced promycelia and sporidia on germination.

Plants of fall panicum (*Panicum dichotomiflorum* Michx.) systemically infected with a smut fungus are abundant at Arlington Experiment Farm, Arlington, Va., each autumn. A healthy and a smutted shoot of this grass, collected in October 1937, are shown in figure 1. The smut fungus agrees well with the description of *Sorosporium*

[1] Received for publication October 11, 1940.

[2] CAMPAGNA, E. "NEW WHITE SMUT" OF WHEAT, COLLECTED AT STE. ANNE DE LA POCATIERE. Quebec Soc. Protect. Plants Ann. Rpt. (1925-26) 18: 71-72. 1926.

[3] HOLTON, C. S. HYBRIDIZATION AND SEGREGATION IN THE OAT SMUTS. Phytopathology 21: 835-842, illus. 1931.

[4] HOLTON, C. S. ORIGIN AND PRODUCTION OF MORPHOLOGIC AND PATHOGENIC STRAINS OF THE OAT SMUT FUNGI BY MUTATION AND HYBRIDIZATION. Jour. Agr. Res. 52: 311-317, illus. 1936.

[5] MOORE, M. B., and ALLISON, C. C. AN ALBINO STRAIN OF BARLEY SMUT. (Abstract) Phytopathology 25: 27-28. 1935.

syntherismae (Pk.) Farl. given by Clinton [6] and by Whetzel and Jackson.[7] Although this smut is reported to occur commonly throughout the eastern part of the United States on various species of *Cenchrus*

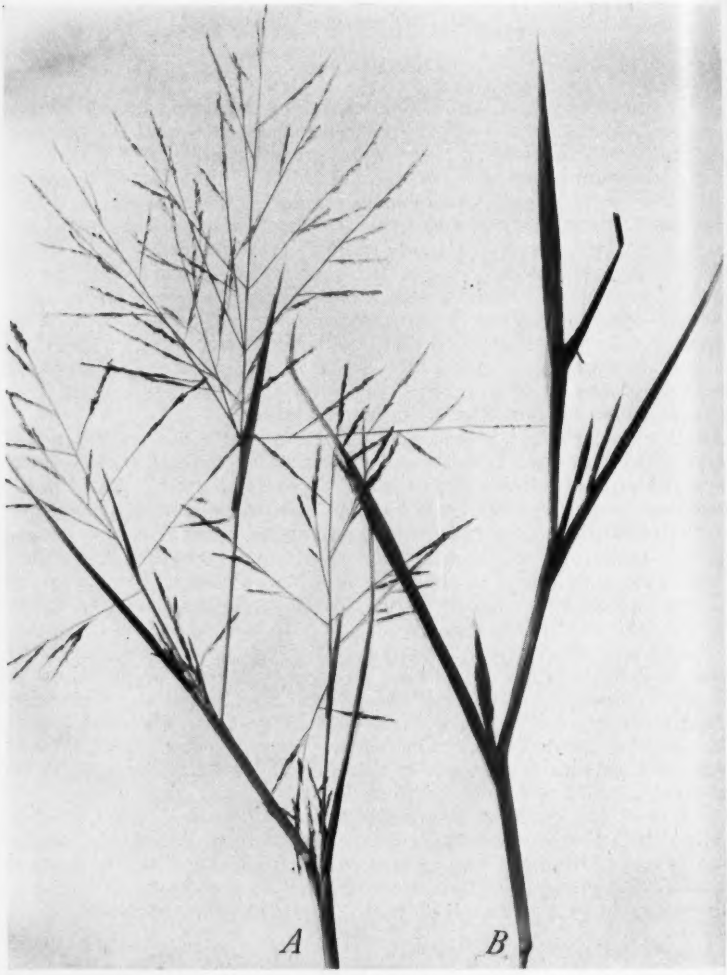

FIGURE 1.—Healthy (*A*) and smutted (*B*) shoots of *Panicum dichotomiflorum* collected at Arlington Experiment Farm, Arlington, Va., in October 1937.

and *Panicum* and is known also from Mexico, South America, and Bermuda, it appeared that little study had been made of it. Consequently a supply of the dark chlamydospore material was collected

[6] CLINTON, GEORGE PERKINS. ORDER USTILAGINALES. North Amer. Flora 7: 1–82. 1906.
[7] WHETZEL, H. H., and JACKSON, H. S. THE RUSTS AND SMUTS OF BERMUDA. Brit. Mycol. Soc. Trans. 13: 1–32. 1928.

and an inoculation experiment was planned to determine the effect of incubation-period temperature on infection.

In the course of the experiment a buff type of smut was observed by the writers. The present paper records the results of an investigation of the pathogenicity of both types and of the relationship between them.

EFFECT OF INCUBATION-PERIOD TEMPERATURE ON PATHOGENICITY OF SOROSPORIUM SYNTHERISMAE

Seed of fall panicum, collected at Arlington farm in October 1937, was dusted with smut chlamydospores (*Sorosporium syntherismae*) on February 23, 1938, and was sown thickly in 4-inch pots of soil. Similarly prepared pots were sown with noninoculated seed as checks, and the pots were placed in pairs in chambers maintained at 5°, 10°, 15°, and 20° C. After 26 days under these conditions, the pots were all assembled on a greenhouse bench. Two weeks later the grass seedlings were transplanted individually to thumb pots and were permitted to grow in these under greenhouse conditions until June 22, 1938, when counts were made to determine the percentages of infection. The results of these counts (table 1) show that after the inoculated seed had been incubated for 26 days at 5°, 10°, 15°, and 20° the percentages of plants developing smut were 70.9, 72.5, 53.3, and 22.2 percent, respectively. The grass seed used in this experiment was not treated previous to inoculation, but only 4 smutted plants developed out of a total of 571 check plants grown from the noninoculated seed (table 1).

TABLE 1.—*Effect of incubation-period temperature on the pathogenicity of Sorosporium syntherismae on Panicum dichotomiflorum at Arlington Experiment Farm, Arlington, Va., in 1938*

Incubation period temperature (° C.)	Plants from inoculated seed			Plants from noninoculated seed		
	Total	Smutted		Total	Smutted	
	Number	*Number*	*Percent*	*Number*	*Number*	*Percent*
5	189	134	70.9	260	1	0.4
10	193	140	72.5	194	2	1.0
15	60	[1] 32	53.3	78	0	.0
20	9	2	22.2	39	1	2.6

[1] 1 smutted plant in this lot had buff sori with hyaline, smooth chlamydospores; all other smutted plants in the experiment had black sori with brown, echinulate chlamydospores.

COMPARISON OF DARK AND BUFF TYPES OF SOROSPORIUM SYNTHERISMAE

MORPHOLOGIC DIFFERENCES

While the counts were being made in the above-described experiment, it was observed that one smutted plant in the inoculated lot incubated at 15° C. had buff sori. The striking difference in the appearance of these two types of smut sori is shown in figure 2, *A* and *B*. The shredding of the host tissue and the balls of hyaline chlamydospores characteristic of the buff-type smut are shown in the enlarged view of a buff sorus (fig. 3).

Microscopic examination of the chlamydospores composing the two types of sori revealed not only a difference in color but also a difference in the spore wall. The spores of the buff sori are hyaline and smooth-walled, while those of the common black sori are brown and echinulate or minutely verrucose. These differences are shown in the photomicrographs (fig. 4, *A* and *B*). The hyaline spores are almost spherical in shape, while the brown spores are subspherical or somewhat

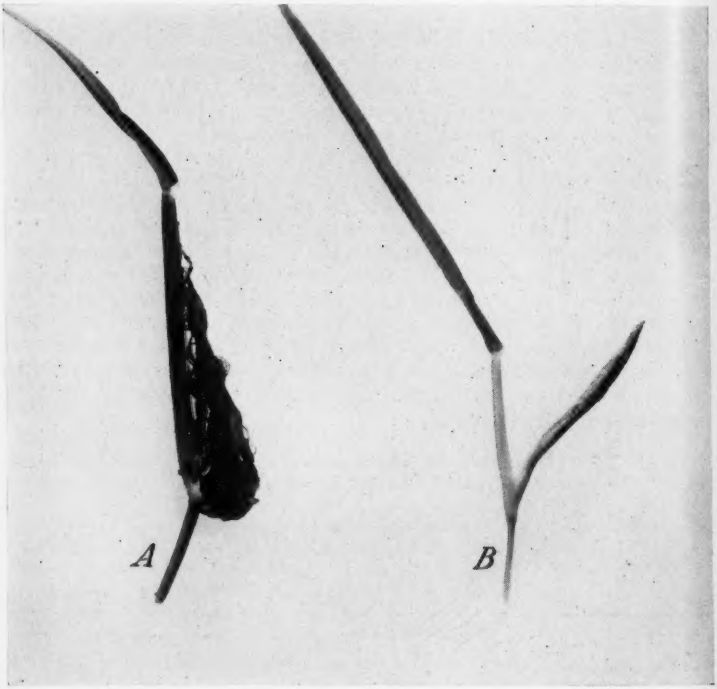

FIGURE 2.—Two types of smut that developed when seed of fall panicum was inoculated with chlamydospores of *Sorosporium syntherismae* collected in the field at Arlington Experiment Farm, Arlington, Va., in 1938: *A*, Common dark-type sorus; *B*, buff-type sorus. Both ×1.

polyhedral. Although the hyaline chlamydospores appear appreciably smaller than the brown when examined under the microscope, measurements failed to reveal any great differences in size. One hundred hyaline chlamydospores had an average diameter of 11.9μ, while a hundred brown chlamydospores averaged 12.7μ by 11.9μ. In a second set of measurements, 25 hyaline spores averaged 11.8μ by 11.5μ, whereas 25 brown spores averaged 12.4μ by 11.5μ. These averages all fall within the 9μ to 13μ spore size recorded by Clinton[8] for *Sorosporium syntherismae*.

[8] See footnote 6.

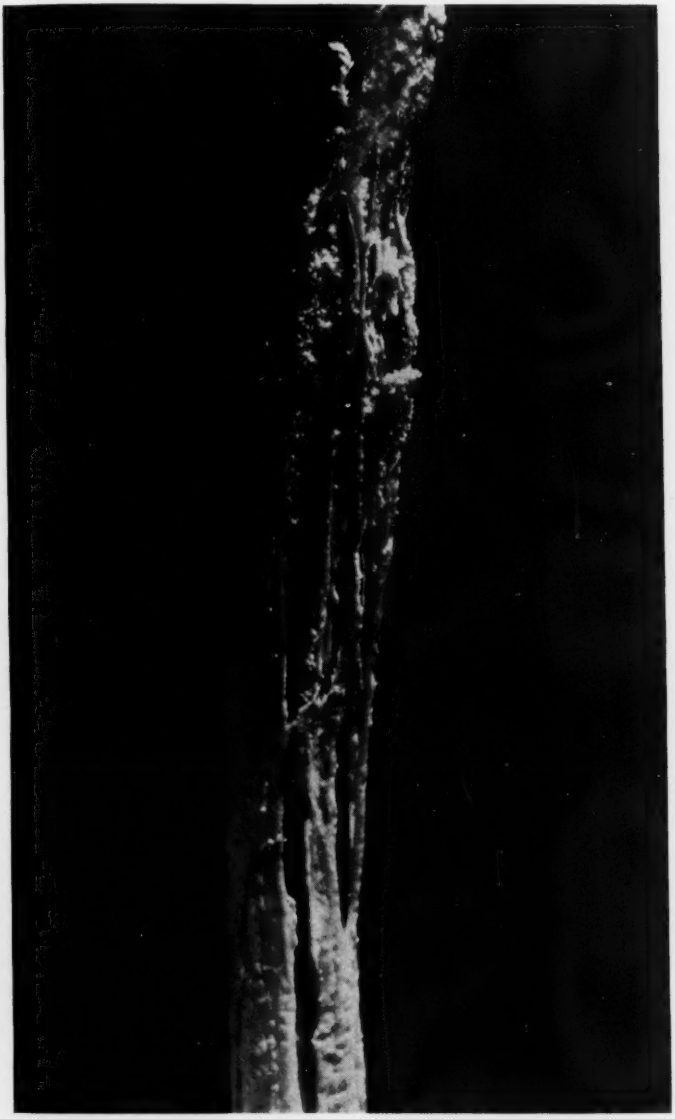

FIGURE 3.—Buff-type sorus of *Sorosporium syntherismae* showing shredding of host tissue and balls of hyaline chlamydospores. ×10.

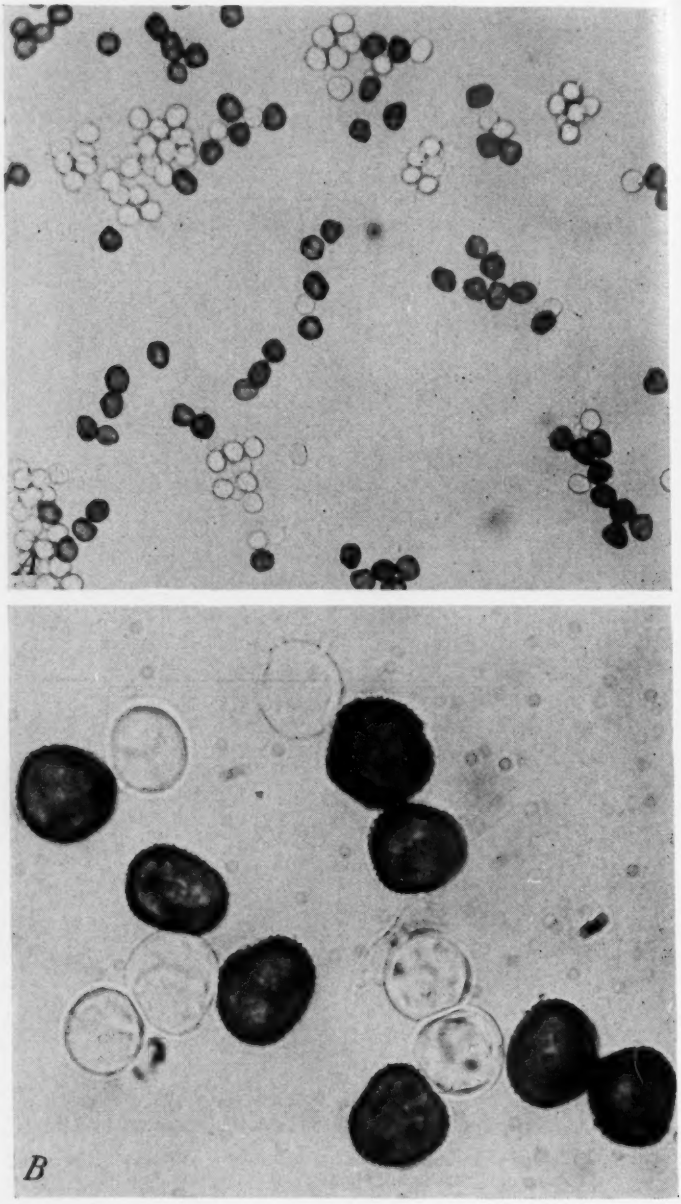

FIGURE 4.—Photomicrographs showing the contrast in color and cell wall between individual chlamydospores of the buff type and of the common dark type of *Sorosporium syntherismae*: *A*, ×350; *B*, ×1,500.

CULTURAL DIFFERENCES

Single chlamydospore cultures of the two types of smut were established and distinct cultural differences were found to exist. The culture of the black smut was of the sporidial type with a pronounced tendency to sector (fig. 5, *A*) whereas the culture of the buff smut was mycelial and quite stable (*B*). To determine the effect of tempera-

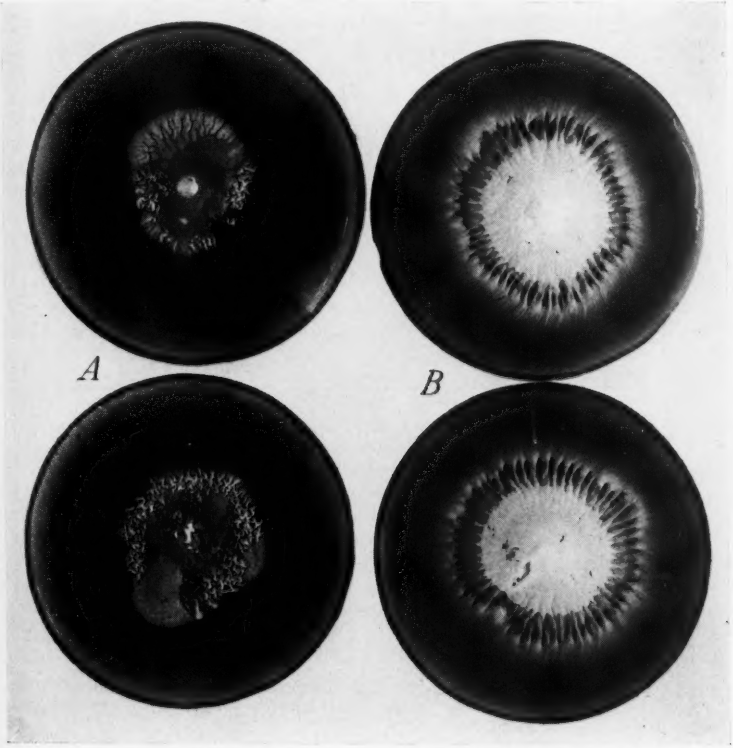

FIGURE 5.—Cultural differences of brown (*A*) and hyaline (*B*) single chlamydospore lines of *Sorosporium syntherismae* grown on potato-dextrose agar for 30 days at 20° C. in 1939.

ture on the growth of a hyaline and a brown single chlamydospore line of *Sorosporium syntherismae*, the fungus was grown on potato-dextrose agar in Erlenmeyer flasks for 30 days. During this period the flasks were located in electric incubators at a temperature range of 5° to 35° C. in 5° intervals.

The average growth in millimeters made by the two types of cultures at each of the seven temperatures employed is shown graphically in figure 6. The similarity in shape of the two curves (fig. 6) shows that the cardinal points for the growth of these two types of cultures are approximately the same. Both grow slowly at 5° C. and have a

distinct optimum near 20° and a maximum somewhere between 30° and 35°. The greater radial growth of the buff smut culture was apparently due to its mycelial nature, while the sporidial type culture of the black smut tended to pile up rather than to spread radially. It is interesting to note that the optimum temperature for the growth of this fungus in culture (20°) is considerably higher than the optimum temperature for infection, which was shown in the inoculation experiment discussed above to be from 5° to 10°.

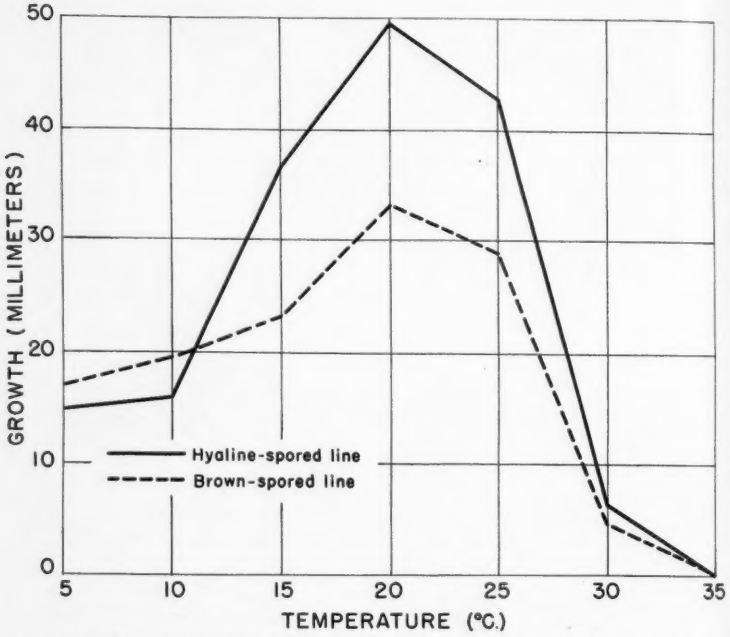

FIGURE 6.—Effect of temperature on the growth of a hyaline and a brown single chlamydospore line of *Sorosporium syntherismae* grown on potato-dextrose agar for 30 days in 1939.

EFFECT OF METHOD OF INOCULATION AND INCUBATION-PERIOD TEMPERATURE ON PATHOGENICITY

On March 6, 1939, an experiment was started to determine the effect of method of inoculation and incubation-period temperature on the pathogenicity of the buff type and of the common dark type of *Sorosporium syntherismae*. On this date seed of fall panicum was given parallel inoculations with the dark-type smut and the buff type. The following methods were used with each type: The seed was (1) dusted with dry chlamydospores, (2) wet with a suspension of chlamydospores, and (3) placed under partial vacuum in a suspension of chlamydospores. The inoculated seed was then planted at once in

4-inch pots of soil, as in the 1938 inoculation experiment, and the pots were placed in chambers maintained at 10° and 20° C. Similarly prepared pots were sown with noninoculated seed as checks and were placed in the same chambers as the treated pots. In the pots held at 20° the seedlings emerged between March 14 and 21, and these pots were transferred to a greenhouse bench during this period. In the pots held at 10° the seedlings had emerged by April 1, and on that date these pots were placed on the greenhouse bench with the others. The incubation period in this experiment thus varied from 8 to 26 days in length as compared with the uniform period of 26 days employed in the 1938 inoculation experiment (p. —).

Three weeks after the final lot of pots was moved from the incubation chambers to the greenhouse, the grass seedlings were transplanted individually to thumb pots and were permitted to grow in these under greenhouse conditions until June 9 to July 17, 1939, during which period counts were made to determine percentages of infection. The results of these counts are summarized in table 2. The data presented in table 2 show that in general a higher percentage of smutted plants developed after an incubation period of 10° C. than after an incubation period of 20°, thus confirming the results of the 1938 experiment. These data show further that dusting the grass seed with dry chlamydospores resulted in more smutted plants than did either of the moist methods of inoculation. It would appear from these data, also, that the common dark type of smut is somewhat more pathogenic than is the buff type, since with the latter type of inoculum percentages of infection were lower in all but one instance. The noninoculated check plants in this experiment all remained free from smut.

The 68 smutted plants which resulted from inoculation of seed with chlamydospores of the buff-type smut all produced only buff sori (fig. 7, *A* and *B*). This is considered further evidence of the stability of the buff-type smut. The 240 smutted plants that resulted from inoculation of seed with chlamydospores of the common dark-type smut all produced only black sori in this experiment. A typical black sorus that resulted from inoculation with dark chlamydospores is illustrated in figure 7, *C*.

TABLE 2.—*Effect of method of inoculation and incubation-period temperature on the pathogenicity of the buff type and the common dark type of Sorosporium syntherismae on Panicum dichotomiflorum at Arlington Experiment Farm, Arlington, Va., in 1939*

Method of inoculating seed	Incubation-period temperature	Plants from seed inoculated with—					
		Buff-type spores			Common dark-type spores		
		Total	Smutted		Total	Smutted	
	° C.	*Number*	*Number*	*Percent*	*Number*	*Number*	*Percent*
Dusted with chlamydospores	10	77	29	37.7	196	167	85.2
	20	59	14	23.7	96	28	29.2
Wet with suspension of chlamydospores	10	165	8	4.9	121	3	2.5
	20	100	2	2.0	69	4	5.8
Partial vacuum, using suspension of chlamydospores	10	67	10	14.9	74	36	48.6
	20	47	5	10.6	99	2	2.0
Noninoculated seed	10	60	0	.0	167	0	.0
	20	53	0	.0	115	0	.0

FIGURE 7.—Buff-type sori (*A* and *B*) and common dark-type sorus (*C*) that developed when seed of fall panicum was inoculated with hyaline, smooth chlamydospores and brown, echinulate chlamydospores, respectively, in 1939. ×1.

SUMMARY AND CONCLUSIONS

An experiment was made at Arlington Experiment Farm, Arlington, Va., in 1938, to determine the effect of incubation-period temperature on the pathogenicity of *Sorosporium syntherismae* (Pk.) Farl. on *Panicum dichotomiflorum* Michx. After seed dusted with chlamydospores had been incubated for 26 days at temperatures of 5°, 10°, 15°, and 20° C., the percentages of smutted plants developing were 70.9, 72.5, 53.3, and 22.2 percent, respectively.

One smutted plant in this experiment was observed to have buff sori composed of hyaline, smooth-walled chlamydospores, while the other 307 smutted plants all had the common black sori composed of brown, echinulate-walled chlamydospores.

Single chlamydospore cultures of the two types of smut were established. The cultures of the buff-type smut were mycelial and stable, while those of the common black-type smut were of the sporidial type with a pronounced tendency to sector. The optimum temperature for the growth of each type on potato-dextrose agar was approximately 20° C., and the maximum for each was between 30° and 35°.

Inoculations made in 1939 with chlamydospores of the buff-type smut resulted in 68 smutted plants, all of which produced only buff sori. Parallel inoculations with chlamydospores of the dark-type smut resulted in 240 smutted plants, all of which produced only black sori.

It is concluded that the buff smut is a result of mutation in *Sorosporium syntherismae* and that the change may involve several genetic factors, since the mutant differs from the common dark smut in spore color, marking of spore wall, spore shape and size to a minor extent, and in degree of pathogenicity.

COMPARISON OF THE WOOL AND SKINS OF FULL-FED AND MAINTENANCE-FED LAMBS [1]

By M. X. SULLIVAN, *research professor of chemistry*, and W. C. HESS, *associate research professor of chemistry, Chemo-Medical Research Institute, Georgetown University, Washington, D. C.*, and PAUL E. HOWE, *chief, Animal Nutrition Division, Bureau of Animal Industry, United States Department of Agriculture*

INTRODUCTION

It has been known since the early work of Buchtala (*6*) [2] that keratin-containing substances, such as wool, hair, nails, horns, and skin, are particularly rich in the sulfur-containing amino acid cystine. As emphasized by Harris and Smith (*11*), the cystine content is, to a high degree, a measure of the stability and quality of wool. Rimington (*16*), in fact, proved that cystine sulfur accounts for practically all the sulfur in wool. This finding has been confirmed by Sullivan and Hess (*18*), who found that the cystine content of the wool tested accounted for 97.1 percent of the total sulfur. However, differences have been observed by Rimington (*16*) in the cystine content of the wool from different breeds of sheep, and also by Ramaiyya (*15*) and Marston (*13*) in the sulfur content and the rate of growth of wool from the same breed of sheep but on different diets. According to Fraser (*10*), the effect of nutrition on wool growth and cystine content is still a moot question.

An opportunity to test the effect of diet on the cystine content of both wool and skins was afforded by a feeding experiment on lambs conducted at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md. The results obtained are reported in this paper.

HANDLING OF THE ANIMALS AND SKINS

The animals used comprised nine pairs of twin Rambouillet wether lambs born at the United States Range Livestock Experiment Station, Miles City, Mont., in the spring of 1933. The lambs were selected for uniformity in age, weight, type, form, grade, and fleece. On the day of weaning (September 15, 1933), the lambs were shipped to Beltsville, Md., where they arrived on September 29. Experimental feeding was begun on October 13. The animals were kept indoors with only slight exposure to direct sunlight but were allowed the run of an outdoor lot for a 2-hour period daily except in stormy weather. They were fed individually, in separate pens for 112 days, a diet consisting of shelled corn, 7 parts by weight; and cottonseed meal, 1 part; corn silage; and clover hay. Water and rock salt were given ad libitum. One animal of each of the nine pairs of twin lambs was full-fed, and the other nine animals received the same diet but in quantities that permitted only a

[1] Received for publication October 3, 1940. A preliminary report on this work in relation to wool was presented before the American Society of Biological Chemists in 1935 (*22*). The authors are indebted to J. I. Hardy of the Bureau of Animal Industry, United States Department of Agriculture, for the sectioning and examination of the fibers according to a technique developed by him, and to F. W. Frey of the Bureau of Chemistry and Soils, now the Bureau of Agricultural Chemistry and Engineering, for the samples of hides that he had prepared for use in a study of the histological structure of the same hides (*7*).

[2] Italic numbers in parentheses refer to Literature Cited, p. 885.

slight increase in weight. A summary of the experimental feeding is shown in table 1.

TABLE 1.—*Average daily feed consumed, body weights, and weights of fleeces and skins of the full-fed and maintenance-fed lambs* [1]

Item	Full-fed lambs	Maintenance-fed lambs
Daily feed consumed:	*Pounds*	*Pounds*
Shelled corn and cottonseed meal (mixture of 7:1)	1.20	0.33
Corn silage	.60	.59
Clover hay	1.01	.56
Salt	.012	.023
Water	3.38	1.54
Initial body weight	47.90	48.00
Final weight after 112 days of feeding	88.70	49.90
Weight of scoured fleece	2.62	1.88
Weight of whole skins	7.88	3.72

[1] Data obtained by V. L. Simmons, of the Bureau of Animal Industry, in connection with the management of the animals.

At the end of the experiment (February 2, 1934) the wool was collected separately from each lamb. If soiled, the samples were "skirted" before being analyzed, that is, soiled edges were sheared. The skins were weighed immediately after removal from the carcass. The left sides were cured, in four piles, with fine new meat salt for 25 days, at the end of which time they contained 14 to 16 percent of salt. All were in good condition except that the top piece of each pile had become dry. Six of the sides, three from the maintenance-fed lambs and three from the twin full-fed lambs, were preserved without further treatment. The skins from the full-fed lambs were thicker and heavier than those from the maintenance-fed lambs; the average weights are given in table 1.

The bend area, or about half of the total area of these skins, was shaved with a razor to remove the wool stubbles. Part of this shaved area was used for the estimation of cystine and sulfur after about 2 years of storage at the Bureau of Chemistry and Soils, now the Bureau of Agricultural Chemistry and Engineering.

EXPERIMENTS INVOLVING CYSTINE AND SULFUR CONTENTS

ANALYTICAL PROCEDURE WITH WOOL

Analyses of the cystine content of the wool and of the cystine and sulfur contents of the skins were made at Georgetown University. For convenience of analytical work the wool from only six paired sets of lambs was taken. Analyses of the various samples (from May 17 to July 24, 1934) were made by the same analyst without any knowledge of the significance of the particular samples.

Before analysis the individual samples of wool were freed from lipoidal matter by means of warm benzol according to the procedure recommended by Rimington (*16*). For the analysis, air-dried samples of the benzol-free water-washed wool were taken of the whole fibers, and also of the whole fibers cut into thirds. The subdivision was made on the assumption that whatever difference might occur as a result of differences in the diets would be manifested in the inner third of the fibers.

The whole fibers were hydrolyzed with 20-percent hydrochloric acid for 6 hours in an oil bath maintained at 125° C. The individual thirds were similarly hydrolyzed. Cystine was determined in the respective hydrolyzates by the Sullivan (*17*), the Okuda (*14*), the Folin-Marenzi (*9*), and the total-sulfur methods, the last mentioned one being determined by the Benedict (*2*) method.

The cystine methods differ in their degree of specificity. The Sullivan method is one of high specificity for free cystine and is negative with cystine tied or altered in any way. This method, in fact, requires that the three groups of cysteine (formed from cystine by reduction) be unsubstituted and in the order as in natural cysteine, $HS—CH_2CH(NH_2)COOH$. It is negative with cystine amine, which contains no carboxyl group, and with homocystine, through which might pass methionine considered to be converted to cystine in the animal body.

The Okuda iodometric method gives a positive result with various disulfides, with cystine congeners in which the S-S linkage is still reducible (such as cystine amine and homocystine), and with non-sulfur compounds of high reducing capacity. In hydrolyzates of purified proteins in general, however, Sullivan and Hess (*18*) have found that the Okuda method closely parallels the Sullivan method.

The Folin-Marenzi method for cystine is less specific than the Okuda or the Sullivan methods and in a number of cases has been found to give too high cystine values (*16, 17, 18*).

ANALYTICAL PROCEDURE WITH SKINS

The salted skins of three pairs of the lambs, after being cut into small pieces (diced), were extracted in a Soxhlet apparatus with ether freed from alcohol and peroxides. The fat-free skins were then extracted with water until they were free from sodium chloride. They were then filtered by suction and dried in a desiccator over calcium chloride.

The skins were hydrolyzed with 20-percent hydrochloric acid for 6 hours in an oil bath maintained at 125° C. The hydrolyzates of the skins contained a considerable quantity of humin, in contrast to the wool hydrolyzates, which contained practically no humin. Accordingly, the skins were hydrolyzed in a reducing atmosphere, that is, with hydrochloric acid containing titanous chloride—a type of hydrolysis that was found by Sullivan (*17*) and by Sullivan and Hess (*19, 21*) to prevent humin formation in a number of proteins. The procedure employed in the present case was as follows: Two grams of each finely cut skin was hydrolyzed for 4 hours with 4 cc. of 20-percent hydrochloric acid and 2 cc. of 20-percent titanous chloride, in an oil bath maintained at 125° C. No humin was observed. The solutions were carefully neutralized with 5 N sodium hydroxide added dropwise with stirring, until no more blue precipitate (titanous hydroxide) occurred. After filtration, part of the filtrate was used for cysteine determination and part for the determination of the total sulfur of the hydrolyzate by the Benedict (*2*) method.

RESULTS WITH WOOL

Table 2 gives the data, obtained by the Sullivan method, for the cystine content of the whole fiber and the various parts of the fiber for each of the animals. Similar analyses were made by the Okuda,

Folin-Marenzi, and total-sulfur procedures. Since all these findings corroborated, in general, those found by the Sullivan method, only the average results are given in table 3.

TABLE 2.—*Cystine content of wool from six pairs of twin lambs, one twin maintenance-fed and the other full-fed (determinations by the Sullivan method)*

Lamb No.	Cystine content of—				Treatment of wool
	Whole fiber	Outer third of fiber	Middle third of fiber	Inner third of fiber	
	Percent	*Percent*	*Percent*	*Percent*	
MAINTENANCE-FED LAMBS					
1344	11.70	11.55	11.77	11.21	Skirted.
1348	11.57	11.96	10.11	8.86	Do.
1430	12.39	12.14	11.10	11.40	Do.
2030	12.05	12.03	11.92	11.13	Unskirted.
2260	11.40	11.49	11.43	11.28	Skirted.
2326	11.49	11.08	11.62	11.47	Do.
Average	11.77	11.71	11.32	10.89	
FULL-FED LAMBS					
1343	11.78	11.02	12.34	11.87	Unskirted.
1349	10.87	10.39	10.90	11.47	Skirted.
1431	12.32	11.66	11.99	12.55	Do.
2029	12.15	11.91	12.15	12.60	Unskirted.
2259	10.88	11.17	11.03	10.42	Skirted.
2327	12.31	10.93	11.64	13.29	Do.
Average	11.72	11.18	11.68	12.03	

TABLE 3.—*Average cystine and sulfur contents of wool from maintenance-fed and full-fed lambs, as determined by various methods*

Method of determining cystine content	Method of feeding lambs	Cystine content of—			
		Whole fiber	Outer third of fiber	Middle third of fiber	Inner third of fiber
		Percent	*Percent*	*Percent*	*Percent*
Sullivan	Maintenance-fed	11.75	11.70	11.32	10.89
	Full-fed	11.72	11.18	11.68	12.03
Okuda	Maintenance-fed	11.94	11.84	11.51	11.13
	Full-fed	11.97	11.39	11.86	12.50
Folin-Marenzi	Maintenance-fed	13.01	12.54	13.32	12.57
	Full-fed	12.37	12.48	13.09	14.51
Total sulfur [1]	Maintenance-fed	12.45	12.24	11.90	11.58
	Full-fed	12.04	11.71	12.01	12.90

[1] The sulfur content of the wool is expressed as cystine by multiplying the value for total sulfur by 3.75, the factor to convert sulfur to cystine.

In the hydrolyzates obtained by the Sullivan method the samples of whole fibers, both the long fibers from the lambs on the full diet and the shorter fibers from those on the maintenance diet, had, on the average, practically the same cystine content. Secondly, in the lambs on the maintenance diet the cystine content tended to decrease from the outer third to the inner third of the fibers, whereas in the full-fed lambs it tended to increase.

The Okuda method gave, in these hydrolyzates, results of the same order of magnitude as the Sullivan method. In the Okuda method,

also, the average cystine contents of the whole fiber of both sets of lambs were practically identical. On the other hand, considering the average cystine content of the inner third of the wool from the lambs on the full ration as 100, that of the inner third from the lambs on the maintenance diet was 89.

In a 6-hour hydrolyzate, the Folin-Marenzi method for cystine frequently gives values higher than that for total sulfur. This result was obtained in the hydrolyzates of the wool from a number of the lambs studied. Despite the impossible high values found in the hydrolyzates of some of these samples of wool, the findings by the Folin-Marenzi method were in general agreement with those of the Okuda and the Sullivan methods. Also, considering the cystine content of the inner third of the wool of the full-fed lambs as 100, that of the inner third of the wool of the maintenance-fed lambs was 87.

Similarly, determining the total sulfur on aliquots of these hydrolyzates and computing this sulfur as cystine, as given in table 3, showed, on the average, practically the same percentages for the whole fibers of the lambs fed the maintenance and full diets. On the other hand, the cystine content of the inner third of the wool from the full-fed lambs and from those on the maintenance diet was in the ratio of 100:90.

Every method of testing showed that the inner third of the wool fibers from the lambs on the full diet was about 10 percent higher in cystine than the inner third of the wool fibers from the lambs on the maintenance diet.

In cross sections of the wool fibers, the diameters of the wool of the maintenance-fed lambs were less than those of the full-fed lambs, but there was no difference in the structure nor evidence of increased medullary space when the fibers were examined either in cross section or longitudinally by polarized light. It appears, therefore, that the lowered cystine content of the inner third of the wool from the maintenance-fed lambs is the result of the change in the protein of the cortex and not an increase of the medullary space and its associated constituents.

RESULTS WITH SKINS

As it was found that the inner third of the wool from the full-fed lambs gave higher cystine and sulfur value than the inner third of the wool from the maintenance-fed lambs, a similar comparison of the relative cystine and sulfur contents was made of the skins of three maintenance-fed and three full-fed lambs. As stated previously, the ordinary hydrolysis with 20-percent hydrochloric acid gave a considerable quantity of humin, which called for decolorization and gave results for cystine slightly lower than when humin formation was prevented. The comparative results, however, were of the same order of magnitude with the hydrochloric acid hydrolysis as with the hydrolysis involving hydrochloric acid containing titanous chloride. Since the latter is considered the better procedure, in the present case only the findings by this method are given in table 4. As determined by iodometric titration before and after reduction with zinc and hydrochloric acid (Okuda method), no cystine was present in the hydrolyzate. With cysteine as the standard the cysteine in the hydrolyzate was determined and computed as cystine.

TABLE 4.—*Cystine and sulfur contents of the skins of maintenance-fed and full-fed lambs*

Lamb No.	Cystine content by—		Total sulfur content in—	
	Sullivan method	Okuda method	Skin	Hydrolyzate
	Percent	*Percent*	*Percent*	*Percent*
MAINTENANCE-FED LAMBS				
1430	0.66	0.67	0.52	0.50
1454	.45	.46	.64	.61
2260	.79	.81	.54	.46
Average	.63	.65	.57	.52
FULL-FED LAMBS				
1431	0.98	1.00	0.75	0.71
1455	.70	.71	.82	.76
2259	.95	1.01	.80	.77
Average	.88	.91	.79	.75

There were distinct differences between animals, but the skins of the lambs on the full diet individually and collectively gave much higher cystine and sulfur values than the skins from the lambs on the maintenance diet. The average ratio of the cystine content of the skins from the lambs on the latter diet to that of the lambs on the former was 100:140 with the Sullivan colorimetric method and the Okuda iodometric method. The ratio of the total sulfur of the skins for the two groups of lambs was, on the average, 100:139. The relative agreement of the two methods shows that no disulfide other than cystine was present.

In the skins the cystine contains only part of the total sulfur. Although there is no information on the methionine content of lambskins, the stratum corneum of human skin has been shown by Wilkerson and Tulane (*29*) to contain at least as much methionine as cystine. The difference between the two groups of experimental animals is brought out more markedly in the skins than in the wool from the lambs on the respective diets.

There are, in biochemical literature, various analyses of the cystine content of human skin. Among these are the following: Abderhalden and Zorn (*1*) for psoriasis scales, 1.85 percent; Wilkerson (*28*) for human epidermal scales, 2.31 percent; Eckstein (*8*) and Block (*4*) 3.82 and 3.40 percent, respectively, for outer layers of human skins. For human skin, Wilson and Lewis (*30*) report a cystine content of 1.8 to 2.3 percent with no details as to whether the skins were entire skins or mainly outer layers.

Since the lambskins utilized in the present work contain closely packed wool roots and included inner and outer skin and connective tissue, it seems a questionable procedure to compare the results with the work of others. However, mention is made of one analysis, by Sullivan and Hess,[3] of a sample of the entire skin obtained from a man accidentally killed. The skin sample freed from fat by acetone and ether extraction, and analyzed by the methods used for the lambskins, gave a cystine value of approximately 0.9 percent, a result comparable with that of the skins from the lambs on the full diet.

[3] Unpublished data.

EXPERIMENTS INVOLVING HISTIDINE, LYSINE, AND ARGININE CONTENTS

Since there was such a marked difference in the contents of cystine and total sulfur in the skins from the twin lambs on the two levels of feeding, it was considered desirable to determine the contents of the basic amino acids histidine, lysine, and arginine. Although several investigators—Wilkerson (*28*), Eckstein (*8*), and Block (*4*)—have reported on these three amino acids in human skin, no work on the basic amino acids of lambskins has come to the present authors' attention. Vickery and Block (*24*) found that wool contained 0.66 percent of histidine, 2.3 percent of lysine, and 7.6 percent of arginine, and later (*25*) reported the molecular ratio of these three amino acids for wool to be 1:4:12.

ANALYTICAL PROCEDURE

Analyses for histidine, lysine, and arginine were made of the whole fibers of the wool from one pair of twin lambs: Lamb No. 1430, on the maintenance diet; and No. 1431, on the full diet. The methods used were those of Vickery and Leavenworth (*26*, *27*), including the modifications introduced by Vickery and Block (*25*) and Block and Vickery (*5*).

Similar analyses were made of the skins of two pairs of the twin lambs. The skins, which, as previously stated, had been preserved in rock salt, were handled as follows: All of them were defatted with anhydrous peroxide-free ether. To determine the effects of the salt on the analytical results, a portion of each skin from one of the two pairs (Nos. 1430 and 1431) was then washed with water to remove the salt, dried in a desiccator over calcium chloride, and hydrolyzed with 8 N sulfuric acid. The remainder of these two skins and the second pair were similarly hydrolyzed without being washed.

The histidine and arginine were isolated as the flavianate and the lysine as the picrate. The percentage of each amino acid was calculated from the weight of the dried flavianate and picrate.

RESULTS

In the wool analyses, the percentages of histidine, lysine, and arginine were 0.71, 2.64, and 9.47, respectively, for the maintenance-fed lamb and 0.71, 2.66, and 9.57 for the full-fed lamb. The molecular ratio of these amino acids is 1:4:12, as found by Vickery and Block. The histidine and lysine contents of the wool were similar to those given by Vickery and Block (*24*). The arginine content was higher than that obtained by these investigators but is in better agreement with that (10.4 percent) reported recently by Vickery (*23*) and obtained by the use of the diflavianate method.

Table 5 gives the results of the analyses of the skin after correction for the moisture and ash in the skin samples.

The table shows that the washed and unwashed samples gave values of the same order of magnitude for histidine, lysine, and arginine. In other words, the presence of 14 to 16 percent of salt in the samples made relatively little difference in the analytical results. Although there is some variation in the percentages of histidine, lysine, and arginine in the skins from the full-fed and maintenance-fed lambs, the molecular ratio is of the same order of magnitude for both pairs of

lambs, being 1:4.4:16 for the unwashed skins of the full-fed lambs and 1:5.2:16 for those of the maintenance-fed lambs.

TABLE 5.—*Histidine, lysine, and arginine contents of the skins of maintenance-fed and full-fed lambs*

Lamb No.	Treatment of skin	Histidine	Lysine	Arginine
		Percent	*Percent*	*Percent*
MAINTENANCE-FED LAMBS				
1430	Washed	0.71	3.36	11.92
	Unwashed	.66	3.12	12.45
2260	Unwashed	.93	3.95	17.40
FULL-FED LAMBS				
1431	Washed	0.73	3.49	11.96
	Unwashed	.74	3.42	13.49
2250	Unwashed	.82	3.36	14.97

Hess (*12*) has shown that the molecular ratio of histidine:lysine:arginine in normal fingernails is of the same order of magnitude (1:6:13) as in arthritic fingernails, whereas, on the average, the cystine content of arthritic fingernails was markedly lower than that of normal fingernails, as reported by Sullivan and Hess (*20*). Much the same result is obtained in lambskins. The cystine and sulfur contents of the skins of the lambs on the maintenance diet were markedly lower than those of the lambs on the full diet. On the other hand, the histidine, lysine, and arginine values are of the same order of magnitude in both sets of skins. Likewise, though the individual skins may differ in the content of the amino acids, the molecular ratios of the basic amino acids histidine, lysine, and arginine are relatively constant. The findings for the skins tend to support the conclusion of Block (*3*) that tissue proteins are built upon or around an "anlage" of relatively fixed proportions of histidine, lysine, and arginine and agree with the ratio found by Wilkerson (*28*) for the stratum corneum of human skin.

SUMMARY

From October 1933 to February 1934, at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md., nine Rambouillet wether lambs, each being one of a pair of twins, were full-fed. The others of the nine pairs of twins received the same diet but in quantities that permitted only a slight gain in weight.

The wool collected separately from six pairs of the lambs was analyzed for cystine, at Georgetown University, Washington, D. C., by the Sullivan, Okuda, Folin-Marenzi, and total-sulfur methods. The sulfur content of all the fibers was likewise determined.

All the methods employed agreed in showing that the wool of the lambs on the maintenance diet contained approximately 10 percent less cystine in the body third of the fibers than did the wool of the lambs receiving the full diet. This reduction was in the protein of the cortex, since examination of the fiber failed to show evidence of increased medullary spaces in the wool fibers of the maintenance-fed lambs.

The cystine and sulfur contents of the skins of three pairs of the lambs were likewise determined. The skins of the lambs on the full-

fed diet contained markedly more cystine and sulfur than those of the lambs on the maintenance diet. The effect of the maintenance diet showed more markedly in the skins than in the wool from the lambs on the two diets.

The data indicate that low levels of feeding influence not only the quantity of wool but also the quality, as measured by the cystine content.

As a result of the marked difference in the cystine and total sulfur contents of the skins from the lambs on the two levels of feeding, determination of the contents of the basic amino acids histidine, lysine, and arginine were made with the skins and wool of several pairs of the lambs. As judged by the findings from two pairs of lambs only a slight variation was found in the contents of these amino acids in the lambskins. The molecular ratio of histidine : lysine : arginine was approximately 1 : 5 : 16. As judged by the analysis of the wool of one pair of lambs the basic amino acids of the wool were of the same order of magnitude for the two levels of feeding. The molecular ratio of histidine : lysine : arginine was 1 : 4 : 12.

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DIGESTIBILITY OF NUTRIENTS IN FOUR VARIETIES OF SWEETCLOVER HAY[1]

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INTRODUCTION

A widespread interest in sweetclover (*Melilotus alba* Desr.) as a pasture and a hay crop with the resultant testing of improved strains and varieties has led to a study of the digestibility of the organic nutrients in four biennial varieties of this legume. Digestion experiments of 10 days' duration, each preceded by a 10-day preliminary period, were carried out with yearling ewe lambs fed sweetclover hay produced in 1937. The first- and second-year crops of Alpha 1, Grundy County, Arctic, and Common White varieties were grown simultaneously during 1936 and 1937 and their chemical composition determined. These studies were designed to measure the chemical and digestibility differences in the four varieties of sweetclover.

DESCRIPTION OF SWEETCLOVER VARIETIES

The following varities were selected for investigation: Alpha 1, a fine-stemmed, low-growing, low-branching, leafy variety sometimes used as an orchard cover crop; Grundy County, a leafy, coarse-stemmed, dwarfed type; Arctic, a tall-growing, high-branching variety with a low leaf percentage; and Common White, a tall-growing, high-branching, coarse strain with a low leaf percentage. Both the first- and second-year crops of each variety were harvested during 1936 and 1937 in order to take account of any differences in climate and soil that might have occurred during the two seasons. A height of 36 inches was taken as the standard at the time of cutting for the first-year stands, and the early bud stage for second-year stands. A 2-inch stubble was left each year.

REVIEW OF LITERATURE

Garber and his coworkers (*2*) [2] show that when second-year sweetclover is cut for hay in the prebud stage it makes a more vigorous recovery than when cut later. They point out that to make the best hays, sweetclover should not be permitted to show flower buds before it is harvested. It is important that the second-year crop be cut so high that a new growth will develop. This is in agreement with the recommendations of Lloyd (*4*).

Willard's experiments (*8*) show that sweetclover hay contains 50 to 60 percent of leaves in the fall of the first year and only 25 to 35 percent during the second year. Hawk [3] reports the leaf yield of the first-year crop to be 47 percent for the Alpha 1 variety, 61 percent for Grundy County, and 47 percent for Common Tall White. Second-

[1] Received for publication June 20, 1940. Published as Scientific Paper No. 463, College of Agriculture and Experiment Station, State College of Washington.

[2] Italic numbers in parentheses refer to Literature Cited, p. 891.

[3] HAWK, V. B. THE EFFECT OF CLIPPING ON THE YIELD AND NITROGEN CONTENT OF SWEETCLOVER. 90 pp. 1938. [Master's thesis. Copy on file Wash. State Col. Libr.]

year crops were found to yield 42 percent for Alpha 1, and 31 percent for Common Tall White.

McMichael [4] has shown that Common White sweetclover should not be cut for hay after the late-bud stage, because in the more mature stages the proportion of fiber to protein increases rapidly. Neidig and Snyder (*5*) studied sweetclover clippings and observed a high dry matter and low protein content in the more mature stages.

At the Ohio Experiment Station (*6*) sweetclover contained an average of 8.13 percent of ash, 17.31 percent of protein, 1.18 percent of calcium, and 0.28 percent of phosphorus on a water-free basis. It ranked well with other legumes in its content of calcium and phosphorus.

Lindsey and his coworkers (*3*) fed green sweetclover to young sheep. The samples of forage averaged 84.5 percent of water, and the dry matter contained 7.08 percent of ash, 19.4 percent of protein, 30.29 percent of fiber, 40.1 percent of nitrogen-free extract, and 3.13 percent of fat. When this clover was fed in the early blossom stage, the sheep digested 69.05 percent of the dry matter, 76.93 percent of the protein, 69.57 percent of the fiber, 69.03 percent of the nitrogen-free extract, and 50.10 percent of the fat. A slightly later stage showed a digestibility of 69.64 percent dry matter, 79.21 percent protein, 49.95 percent fiber, 68.48 percent nitrogen-free extract, and 52.28 percent fat. Results of tests with the later stage showed a marked decrease in digestibility of crude fiber. The experiment showed a high utilization of the sweetclover nutrients in a stage just previous to blooming.

CHEMICAL COMPOSITION OF FORAGE SAMPLES

The composition of the water-free samples is shown in table 1. The moisture in the air-dry samples is also indicated. First and second-year Grundy County samples were higher in protein than the other varieties. In all but one instance the second-year samples contained more crude fiber. The calcium and phosphorus figures are very similar to those previously reported by the author (*7*) for alfalfa hay.

TABLE 1.—*Composition of water-free samples of sweetclover hay of four varieties harvested in 1936 and 1937*

Crop and variety	Year's growth	Moisture in air-dry samples	Chemical composition of dry matter						
			Ash	Crude protein (N×6.25)	Crude fiber	N-free extract	Ether extract	Calcium	Phosphorus
1936 crop:		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Alpha 1	First	6.47	8.65	13.82	34.09	41.28	2.16	1.26	0.31
	Second	7.92	8.93	15.73	30.85	42.36	2.13	2.37	.32
Grundy County	First	6.73	10.09	17.05	26.72	43.02	3.12	1.80	.35
	Second	6.93	8.94	16.83	33.53	39.03	1.67	1.59	.32
Arctic	First	6.48	8.97	14.83	31.14	42.36	2.70	1.65	.32
	Second	7.21	8.68	14.48	38.40	37.01	1.43	1.56	.30
Common White	First	6.76	9.57	15.69	29.93	42.85	1.96	1.68	.32
	Second	7.34	8.87	15.59	31.69	40.66	3.19	1.87	.31
1937 crop:									
Alpha 1	First	16.81	9.17	15.00	32.10	41.00	2.73	.....	.....
	Second	17.51	9.40	14.28	33.69	40.46	2.17	.....	.....
Grundy County	First	19.57	9.31	15.57	29.65	42.77	2.70	.....	.....
	Second	19.73	9.61	17.78	29.75	40.90	1.96	.....	.....
Arctic	First	16.57	9.76	14.56	30.82	42.26	2.60	.....	.....
	Second	17.15	8.91	14.16	38.14	37.14	1.65	.....	.....
Common White	First	18.29	9.48	14.81	32.76	40.36	2.59	.....	.....
	Second	23.46	8.69	14.66	33.96	40.87	1.82	.....	.....

[4] MCMICHAEL, S. C. THE AGRONOMIC VALUE OF VARIETIES OF BIENNIAL WHITE SWEETCLOVER. 66 pp. 1932. [Master's thesis. Copy on file Wash. State Col. Libr.]

TABLE 2.—*The apparent digestibility of 4 varieties of sweetclover hay when fed to lambs*

Variety	Year's growth	Lamb No.	Live weight of lambs		Daily dry-matter intake	Apparent digestibility of—					Additional data for 1937 crop computed to a 10 percent moisture basis	
			Initial	Final		Dry matter	Protein	Crude fiber	N-free extract	Ether extract	Digestibility of crude protein	Total digestibility of nutrients
			Kilograms	*Kilograms*	*Grams*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Alpha 1	First	1	36.7	34.9	255.4	43	65	40	55	31		
		2	45.4	44.4	490.1	57	74	41	67	43		
		3	49.4	49.4	711.3	56	73	40	66	40		
		Average				52.0	70.7	40.3	62.7	38.0	8.80	46.13
	Second	1	34.9	35.1	490.2	55	65	47	63	31		
		2	45.1	44.4	494.9	55	67	46	63	27		
		3	48.8	49.2	705.3	54	67	45	62	20		
		Average				54.7	66.3	46.0	62.7	26.0	9.38	47.18
Grundy County White	First	4	51.3	50.3	667.0	58	72	47	67	42		
		5	49.7	50.8	649.2	57	70	43	67	45		
		6	54.9	52.6	679.6	57	72	42	67	46		
		Average				57.3	71.3	44.0	67.0	44.3	10.94	50.25
	Second	4	49.9	49.9	665.7	62	74	51	70	29		
		5	54.9	53.5	678.3	58	73	45	66	29		
		6	50.4	50.6	647.9	56	70	40	65	30		
		Average				58.7	72.3	45.3	67.0	29.3	10.95	49.30
Arctic	First	4	49.4	49.9	688.3	60	74	45	69	44		
		5	53.1	52.6	700.8	57	73	42	67	38		
		6	51.0	51.0	675.8	57	73	42	68	40		
		Average				58.0	73.3	43.0	68.0	40.7	9.78	50.01
	Second	4	47.8	49.7	683.5	56	66	52	61	21		
		5	52.8	52.8	695.9	53	67	45	59	28		
		6	49.9	51.5	671.1	53	65	47	57	28		
		Average				54.0	66.0	48.0	59.0	25.7	8.61	45.59
Common White	First	1	39.2	37.2	465.7	53	71	37	61	44		
		5	47.6	45.4	488.5	55	73	40	63	45		
		3	49.0	47.8	699.8	57	73	45	67	39		
		Average				55.0	72.3	40.7	63.7	42.7	10.21	47.44
	Second	1	37.2	36.1	425.3	50	62	41	59	7		
		2	46.3	44.7	457.6	48	65	35	58	20		
		3	49.9	48.5	655.5	49	64	38	58	11		
		Average				49.0	63.7	38.0	58.3	12.7	8.93	41.94

DIGESTION EXPERIMENTS

Records of sheep weights and feed intake are summarized in table 2. The same group of lambs was fed both the first- and second-year forage samples of any particular variety in the digestion experiments in order to equalize individual differences. The dry-matter intake during each trial for each lamb in any series was approximately the same (with the exception of lamb 1, trial 1) so that any differences in digestibility that might be attributed to unequal feed intake were reduced to a minimum. The records show quite a uniform relationship of water to dry-matter intake. The protein in the feces ranged from

4.07 to 6.71 percent, the dry matter from 38.40 to 64.10 percent, and the ash from 4.25 to 7.96 percent.

The coefficients of apparent digestibility obtained from 24 individual digestion experiments with lambs are contained in table 2. Averages computed for each trial were used in computing the digestible nutrients of the forages studied.

The results with Alpha 1 sweetclover show a higher digestibility of protein and fat in the first-year hay. The fiber and dry matter were digested slightly better in the second-year hay, no differences being indicated for the nitrogen-free extract.

With the exception of fat, which was digested better in the first-year crop, the nutrients of Grundy County sweetclover were as well digested in the first-year as in the second-year growth.

Arctic sweetclover showed a higher digestibility of dry matter, protein, nitrogen-free extract, and fat in the first-year growth than in that of the second year. The reverse relation was true for crude fiber.

Common White sweetclover of the first-year growth had higher coefficients of apparent digestibility for dry matter, protein, nitrogen-free extract, and fat, than those obtained by feeding the second-year crop.

Grundy County sweetclover appeared to be the only variety studied in which the nutrients of the first-year growth were not digested better than those of the second-year growth.

The percentages of digestible crude protein and total digestible nutrients in the eight samples of hay fed during the digestion experiments are contained in table 2. In order to facilitate comparisons of varieties on a more practical basis, the composition of the forage samples was adjusted to a 10-percent moisture content.

In 1934 the author (7) reported average results for three cuttings of northern-grown, common alfalfa, containing 10 percent of moisture. The digestible protein totaled 9.77 percent and the total digestible nutrients 48.43 percent as determined with sheep. A comparison of the eight samples of sweetclover hay with the nutrients in alfalfa (*Medicago sativa* L.) valued as 100 percent is shown in table 3.

TABLE 3.—*Average digestibility of protein and total digestible nutrients in 3 cuttings of northern-grown alfalfa hay as compared with averages for 8 samples of sweetclover hay of 4 varieties.*

[Values for alfalfa=100]

Hay	Year's growth	Protein	Total digestible nutrients
Alfalfa (northern-grown, common)		100.0	100.0
Sweetclover variety:			
Alpha 1	First	90.1	95.3
	Second	96.0	97.4
Grundy County	First	112.0	103.8
	Second	112.1	101.8
Arctic	First	100.1	103.3
	Second	88.1	94.1
Common White	First	104.5	98.0
	Second	91.4	86.6

The table shows second-year Common White sweetclover to be the lowest in total digestible nutrients of the eight forage samples.

SUMMARY AND CONCLUSIONS

First- and second-year crops of four biennial varieties of sweetclover were studied in digestion experiments with lambs.

The nutrients of Alpha 1 and of Grundy County White were as well digested in the first- as in the second-year hays. Second-year Arctic had a lower digestibility of dry-matter, protein, nitrogen-free extract, and fat than did the first-year sample. First-year Common White was superior to the second-year crop because the protein and especially the crude fat were better digested. The digestible protein of the eight forages reduced to a 10-percent moisture basis ranged from 8.6 to 10.95 percent, and the total digestible nutrients from 41.94 to 50.25 percent.

With respect to digestible protein the hays ranked in the following order: Second-year Grundy County, first-year Grundy County, first-year Common White, first-year Arctic, second-year Alpha 1, second-year Common White, first-year Alpha 1, and second-year Arctic.

The ratings in respect to total digestible nutrients were as follows: First-year Grundy County, first-year Arctic, second-year Grundy County, first-year Common White, second-year Alpha 1, first-year Alpha 1, second-year Arctic, and second-year Common White. In each case first-year crops occupy three of the first four places. The sweetclover hays contained from 88.1 to 112.1 percent as much digestible crude protein and 86.6 to 103.8 percent as much total digestible nutrients as a good grade of alfalfa hay.

First- and second-year Grundy County, first-year Arctic, and first-year Common White were the ranking varieties in regard to feed value when both protein and total digestible nutrients were considered.

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